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miRNA as biomarker for body-fluids identification

Its role in Forensic Sciences

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It is impossible to say how first the idea entered my brain; but once conceived, it haunted me day and night

Edgar A. Poe

Acknowledgment

No one should brave the underworld alone.

Edgar A. Poe

So many persons got my back and give me such support throughout this extraordinary chapter in my life. I cannot afford to acknowledge all the surprising individuals that were there to help me or motivate me - there is why I do want to let you know that I am thankful for making this dissertation possible.

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Pour les deux personnes les plus importantes dans ma vie, à ma maman et à mon papa…

Pour tout ce que vous faites pour moi

Pour tous ce que je suis grâce à vous

Pour tout ce que vous représenté pour moi …

Thank you

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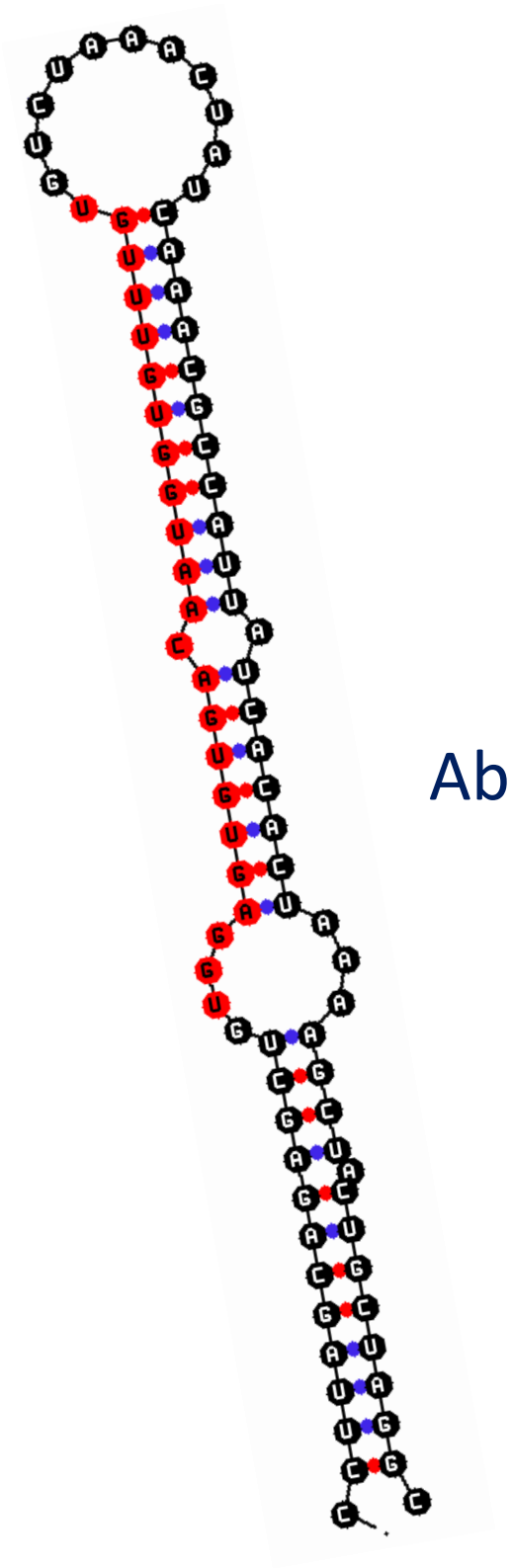


Abstract

Abstract

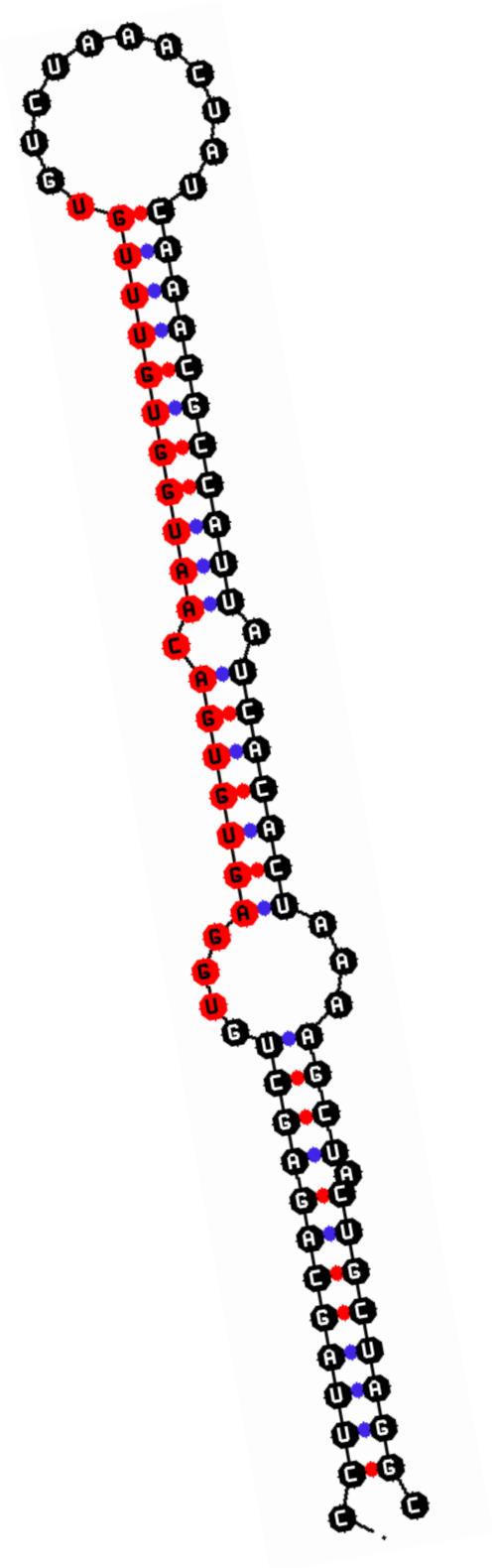
In forensic investigation, body fluids represent an important support to professionals when detected, collected and correctly identified. Through many years, various approaches were used, namely serology-based methodologies however, their lack of sensitivity and specificity became difficult to set aside. In order to sidetrack the problem, miRNA profiling surged with a real potential to be used to identify evidences like urine, blood, menstrual blood, saliva, semen and vaginal secretions.

miRNAs are small RNA structures with 20-25nt whose proprieties makes them less prone to degradation processes when compared to mRNA which is extremely important once, in a crime scene, biological evidences might be exposed to several unfavourable environmental factors.

First of all, we proceed to an extensive gathering of information published till date and assessed a multitude of factors that have a potential aptitude to discrediting miRNA profiling, such as: methodological approaches, environmental factors, physiological conditions, gender, pathologies and samples storage.

Afterwards, we studied 4 miRNAs profiles within 2 body fluids – blood and urine – and settled whether or not those could be used as biomarkers for blood and urine identification.

Keys words: Forensic Science, Body fluids, Profiling, miRNA, Biological biomarkers



Resumo

Resumo

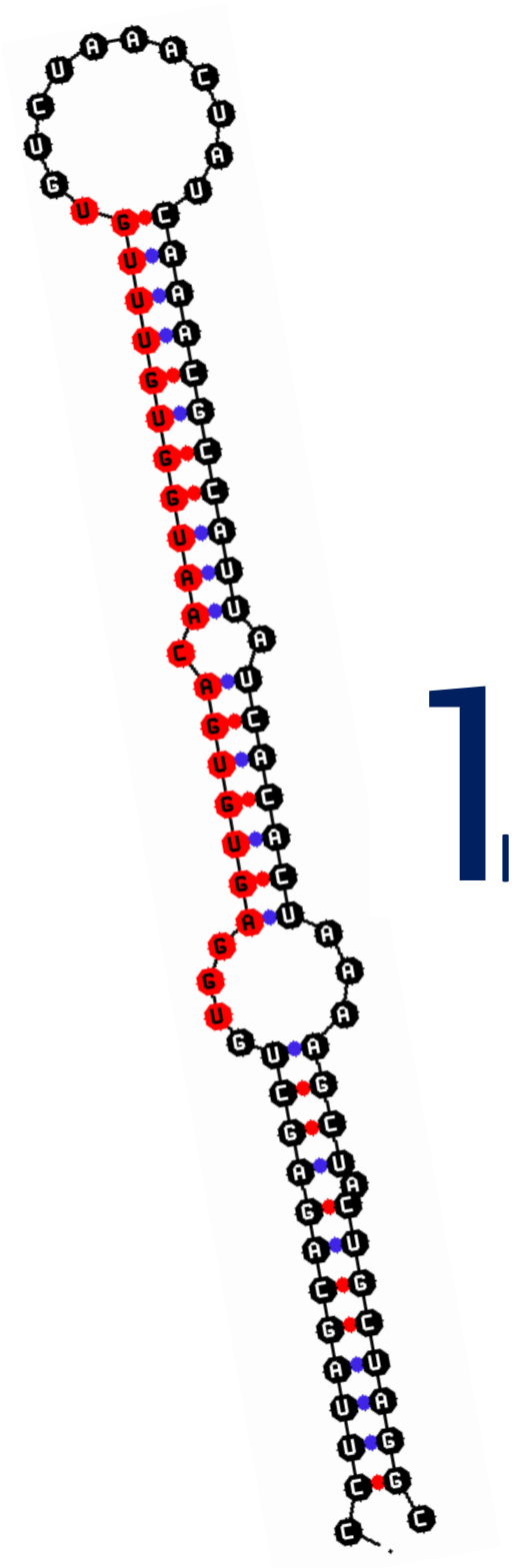
Na investigação forense, os fluidos corporais representam um importante apoio para os profissionais quando detetados, coletados e corretamente identificados. Ao longo dos anos, várias metodologias foram abordadas nomeadamente tecnologias assentes em técnicas serológicas, no entanto, a sua falta de sensibilidade e especificidade tornou-se uma menos-valia difícil de contornar. De forma a dissipar o problema, o estudo dos perfis dos miRNAs surgiu com um verdadeiro potencial para identificar evidências tais como urina, sangue, sangue menstrual, saliva, sémen e secreções vaginais.

Os miRNAs são pequenas estruturas de RNA com 20-25nt cujas propriedades os torna menos propensos a processos de degradação quando comparado ao mRNA, o que é extremamente importante uma vez que, numa cena de crime, as evidências biológicas podem estar expostas a vários fatores ambientais desfavoráveis.

Em primeiro lugar, procedeu-se a uma extensa revisão bibliográfica publicada até a data e avaliou-se uma variedade de fatores com potencial para descreditar o uso de miRNA para a identificação de fluidos biológicos, tais como: uso de diferentes metodologias, fatores ambientais, condições fisiológicas, género, patologias e acondicionamento de amostras.

Em seguida, estudou-se o perfil de 4 miRNAs em 2 fluidos corporais - sangue e urina – com intuito de concluir se estes poderão ser ou não utilizados como biomarcadores para uma identificação fiável de sangue e urina.

Palavras-chave: Ciências Forenses, Fluidos Biológicos, *Profiling*, miRNAs, biomarcadores.



1 Introduction

1 - Introduction

1.1 - Analytical mind

It will be found, in fact, that the ingenious are always fanciful, and the truly imaginative never otherwise than analytic.

Edgar A. Poe

Edgar Allan Poe, master of gruesome plots, released in 1841 the mysterious short-story "The Murders in the Rue Morgue". Poe created as his main character, Auguste Lupin whose, with no other than his mind and incomparable confidence, hunted clues using each one of his senses in order to solve unsolvable crimes. “The murders in the Rue Morgue” became the first detective fiction gender becoming a pillar to other works like Arthur Conan Doyle’s *Sherlock Holmes*.

Before human behaviour was recognized as an important part in criminal investigation, Poe narrated how the body language of a suspect, his accelerated heartbeat, rapid breathing and his guilt as a powerful tool to understand someone’s blame in a crime. The way he conducted his story, based in analytical thinking, “behavioural profiling” and intelligence, led the readers to participate alongside with the main character to solve crimes.

Edgar Allan Poe’s work projected forensic investigation to a wider public. With his books, Poe described the premises of scientific investigation through the sharp mind of his main character. Culminating, centuries later, in one of the most respected and fascinating science of all.

1.2 - Forensic Sciences

Even in the grave, all is not lost.
Edgar A. Poe

The term “forensics” is a Latin word that means “belonging to the forum” - in ancient Rome, if a criminal case was brought up, it will be brought up before the “forum”. Constituted by witnesses and specialists, they will discuss the situation with great detail in order to determine guilt in civil or criminal disputes and persuade the forum of it [1].

Nowadays, forensic science could be defined as the application of a broad spectrum of scientific fields responsible for answer questions of interest in a legal system whether in civil or criminal actions [2]. Modern forensic science investigation, thrived by Locard’s principle - *every contact leaves a trace* - is capable to apply principles but also scientific techniques to analyse evidences recovered during a criminal investigation [3, 4]. It is also based on strict guidelines in order to ensure cautious and methodical collection, organization and analysis of information [5].

Forensic science can be dissected into several specialized divisions namely forensic pathology, anthropology, entomology, odontology, toxicology and genetics. Each with its own set of technologies, they contributes to solving crimes through investigative activities like determining the cause of death, finding missing persons, identifying suspects and profiling criminals [6-13].

1.3 - Evolution of Forensic Science

If you run out of ideas follow the road; you'll get there

Edgar A. Poe

It is commonly though that forensic science is a relatively new science in our society. Far behind the glamorous ideology of the 21th century TV series, criminal investigation is definitely not such a recent science as it may appear. The beginning of Forensic sciences, what we could call - pre-modern forensic science

– was not exactly forensic science. Before the 17th century, for the most part of it, it was not "real" forensic science, however, some of its background did develop on that epoch. By then, for crimes as murder or rape, there would be the confrontation of the accuser and whoever is being accused of that crime. The suspect would be tortured until its confession - which will ensure its guilt. On the other hand, if the suspect was able to resist the pain and have the strength to go through the torture it would be considered as innocent [14].

A pre-historic drowning of a hand with ridge patterns found in Nova Scotia and imprinted fingerprint in clay tablets used for business transactions in ancient Babylon, seem to point the origin of dactiloscopy for common use at 1000-2000 Before Christ [15-17]. Around year 250BC, a Greek physician, Erasistratus, observe that his patient pulse rates increased when he lied. His observations were considered as what would be the first lie detection test [18].

In 44 BC, Antistius, the personal physician of Julius Ceasar, realized the first recorded autopsy known. He concluded that even though Ceasar was stabbed 23 times, the second blow of the knife – the one that hit his heart – was the one that killed him [19, 20].

On the 3rd century, in China, *Yi Yu Ji* was published and has some semblance of a science applied to criminal cases. One of the cases regarded in that publication was of a man that burned to death. His wife claimed that it was an accident, however, officials found that there was no ashes in his mouth which raised their suspicion. In order to elucidate their doubt, their burned 2 pigs, one alive and another already dead. The pig that was alive when burned has ashes in his mouth; on the other hand, the pig that was dead did not. Upon this fact, it was proven the culpability of the victim's wife [21].

In 1248, still in China, was published the first forensic science book called as *Hsi Duan Yu* (the washing away the wrongs). This book discussed the differences between crimes as strangulation *versus* drowning and became an official manuscript for physicians [22-24]. Still that year, a man was stabbed to death with a sickle. Sung Tz'u, a medical examiner, gathered everyone in the village and had them laid their knives and sickle on the ground and waited. Over time, flies came and landed in a particular sickle distinguishing, among others, the murder weapon and by association, the perpetuator of the crime [25, 26].

The German *Constitutio Bambergensis Criminalis,* appeared in 1507, that highlighted the importance of physicians in cases of infanticides [27]. Also known

as the Carolina, it was recognizes as the first criminal law body and punished actions as murder, robbery, manslaughter among others [28].

The *Father of surgery*, Ambroise Paré, wrote and published in 1575 *Reports in Court*. The French surgeon, already known for his studies on death wounds (pre and post mortem), infanticides, hanging, among others, launch through his manuscript a new era – The era of modern forensic pathology [29, 30].

Hydriotaphia, Urn Burial, wrote by Sir Thomas Browne in 1658 make for the first time reference to adipocere [31]. The English physician, also known as a pioneering forensic archaeologist, described the unknown process as a soap-like substance related with moist places. Although, the term adipocere will only surge in 1789 by Antoine François Fourcroy [32].

In 1687, Marcello Malpighi noticed the ridges, spirals and loops on fingertips, which would become fundamental for human identification [15, 33, 34]. However he did not acknowledge their function or importance as a mean of human identification [34].

In 1775, the Swedish chemist Carl Wilhem Scheele created a test able to detect arsenic poison in corpses [35, 36]. His work would be improved by Valentin Rose, who will discover a far more precise method to detect small amounts of arsenic [37].

Three years later, the first recognized documented use of physical matching – bullet wound to a suspect - was released in UK. John Tom was convicted for the murder of Edward Culshaw after a wad of paper found in the victim wound was complementary with the missing corner of the sheet in the possession of the suspect [38].

In Paris, the former convict Eugène François Vidocq created the first detective force in 1810 [39, 40]. The so-called Sureté of Paris was staffed with criminals and was specialized in criminal investigation [41]. His work established Vidocq as the father of modern criminology [41]. The Vidocq Society, created in honor of Vidocq is composed by forensic professionals and private citizens who directed their work to solve cold cases [42].

1813 unveiled one of the most important individual in forensic toxicology – Mathieu Orfila [43]. Considered as the father of modern toxicology, Orfila published *Traité des poisons* which was the first report on poison detection and started to be used as a guideline for murder cases [44].

In 1828, William Nicol invented the polarized light microscope that led Henri-Louis Bayard in 1839 to formulate the first procedures for microscopic detection of sperm [45-47]. Later that year, James Marsh, a Scottish Chemist, was the first person to testify using toxicological evidences in a criminal trial [22, 48, 49]. That is when we start to see the merging between science and law - which is the principle of forensic science.

1850s to 1860s settled for a development of photography which improved records in forensic science more specifically in prison system [50].

In 1879, the French anthropologist Alphonse Bertillon introduced the *Bertillon system* which is also known as anthropometry [51]. He used a large amount of measurements of the body to identify people by their physical appearance [52, 53]. However, this worked relatively well until a particular case – the *Will West case* – put it to an end (figure 1). In 1903, a man called Will West was put in prison. The person who was processing him asked if Will West had already spent time in prison before which he answered negatively. The clerk went to the record and founds out that there was another person in that prison by the name of William West that looked nearly identical. When put in the *Bertillon system,* their measurements were also practically identical. This coincidence put an end to the commonly used method of identification [54-56].

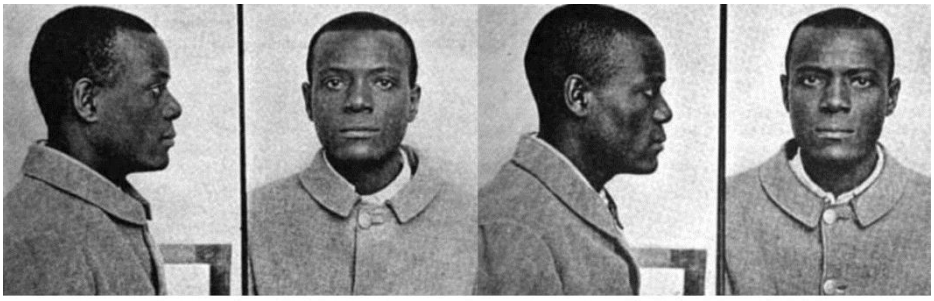


Figure 1 *– The West Case* - Photographs of both Will and William West displaying their undeniable resemblance.

Hans Gross, in 1893, published *Criminal Investigation* that discussed the benefits of botany, chemistry, fingerprinting, geology, microscopy, physics and zoology in criminal investigations [57, 58].

The Nobel Prize winner Karl Landsteiner discovered in 1901 human blood groups which would be adapted later on by Max Richter for blood stains typing [59-62].

The American President, Theodore Roosevelt, established in 1905 the Federal Bureau of Investigation (FBI) whereas FBI director J. Edgar Hoover opens the FBI laboratory in 1934 [63, 64].

In 1910, Albert Osborn published *Questioned Documents* which is still a reference when determining document forgeries [65, 66].

Another important personality in the forensic field is Edmond Locard. Between 1877 and 1966 the French doctor and criminologist opened the very first crime laboratory in France but most importantly, he created the Locard's Exchange principle. Basically, this principle relies on the fact that anytime 2 things make contact, there is an exchange of material [67, 68]. There was a case involving possible forged coins that had 3 suspects. Locard asked to see the suspect's clothes that they were wearing when arrested and was able to find small metal fragments on them. Locard match those metal fragments to the forged coins resulting in the condemnation of the suspects [69].

The American chemist Walter McCrone, between 1916-2002, was the leading expert in microscopy and was the one asked to examined the famous Shroud of Turin and the Vinland map [70-72].

In 1990, the FBI created its personal database of genetic profiles from convicted criminals and those associated with unsolved crimes. Named *Combined DNA Index System* – CODIS – the program constitute a real step forward in forensic genetic field (figure 2) [48, 73].

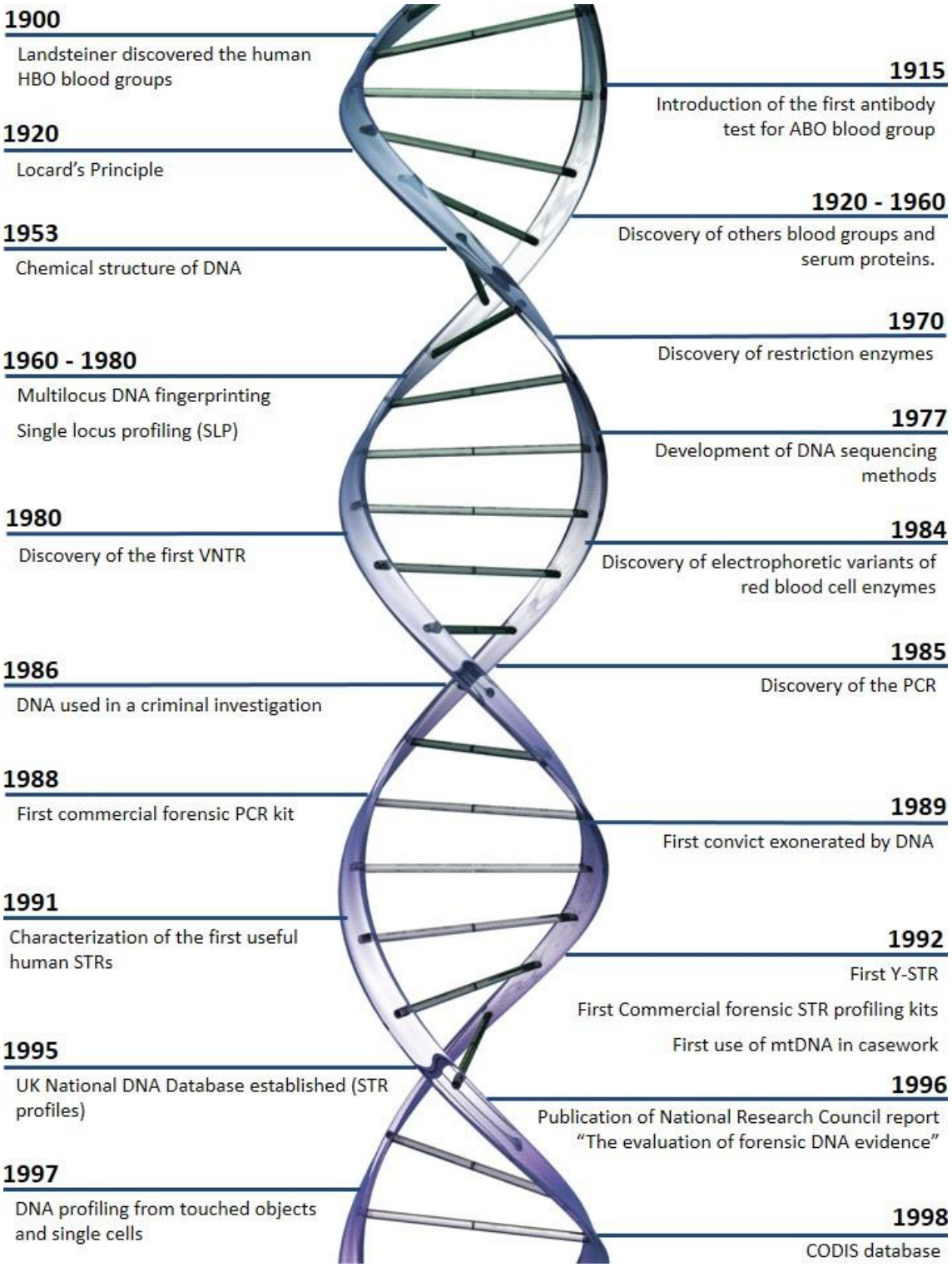


***Figure 2** – Timeline of the major developments in forensic genetics.*

1.4 - Forensic Genetics

Invisible things are the only realities

Edgar A. Poe

1.4.1 - Study Case

In 1987, days after Thanksgiving, the detective Joe Horgas was on duty when he received a call for a homicide case in Arlington suburb, outside Washington D.C. He and the other officers proceeded through the house and found the dead body of the 44 years old Susan Tucker laid face down on the bed of the master bedroom. Naked with just a blue sleeping bag next to her, she had her hands tied behind her back with a brand-new rope going from her hands up to her neck. By the time of the murder, Susan's husband was in Great Britain visiting his family in Wales, where the couple has been planning to move. Agent John Coale and Rick Schoembs from Arlington County police department investigate the crime scene and documented it with close-up photography. On the floor nearby, an empty purse with its content scattered on the floor. The officers zeroed-in what they thought to be the point of entry of the assailant. Outside of the house there was a patio and just beneath it a narrow basement window that was found broken. The officers collected all the broken glass they could find. It is important to acknowledge that there is always a transfer of material between 2 objects – Locard's Exchange principle. If the assailant did entry the house by that window, the glass would present some kind of evidence occasioned by an exchange of assailant-glass material.

Curiously, a similar murder occurred 3 years earlier. Carolyn Hamm was raped and murdered only 3 blocks away from Susan Tucker's house. Just as Susan Tucker case, the assailer entered her house by a narrow opening and the content of her purse was scattered on the floor yet nothing of value was missing. Because of it, Joe Horgas pulled the Carolyn Hamm file in order to get leads to his case. Carolyn Hamm's file was closed, a man was convicted for her murder and already behind bars but doubts still remained. Everyone who worked in the case didn't believe that the suspect – David Vasquez – was responsible for the crime or at least, it was not the only responsible. David Vasquez was serving 34 years of reclusion, he plead guilty in order to avoid death penalty. Then, 3 years later, the same exact crime happened. Detective Joe Horgas believed that the perpetuator of Carolyn Hamm's murder was the same of Susan Tucker's. Despite the

similarities, Horgas had no leads and any fingerprints were found. However, Horgas had hopes for the other evidences. Once in the laboratory, the blue sleeping bag, Susan's nightgown and a washcloth with smears on it were analysed and it was found 4 different stains just in the nightgown. They tested them with acid phosphatase and the purple colour indicated the presence of semen but to confirm it, a small portion of the stain was examined microscopically. After positively identified, remained the questions: to whom it belongs? In 1987 the only way to connect semen to a rapist was, first of all, by determining if any blood was present in it. Is so, the blood could be typed. In that case, the blood type was O and matched neither the victim nor her husband. The results narrow down the field of possible suspects, however, the stained washcloth held an additional piece of the puzzle – 2 pubic hairs. The pigments granules analysis enabled the determination of the race of the suspect and in that case, the pubic hair came from a black man.

At the same time, in Richmond (2 hours from Harlington), a serial murderer known as the South Side strangler was on the loose. Responsible for the rape and murder of 3 women within the preceding 3 month, could the South Side strangler be responsible for the murders in Arlington? Detective Horgas went to Richmond to compare notes and the similarities were impressive. All 3 victims were tied-up and strangled but most importantly, in all cases, the assailant entered by the window. Later, lab tests confirmed that the semen stains found in the 3 murders were identical in blood type to those found in Susan Tucker's nightgown. However, a common blood type is hardly enough to implicate a killer.

In 1987, Deoxyribonucleic acid (DNA) testing was not widely used in forensic labs. Detective Horgas sent the semen stains found in the nightgown of Susan Tucker to a laboratory in New York. Richmond police also submitted semen samples from the 3 murders. Back then, the process had a limited sensitivity, it was required a large amount of biologic sample and its analytic study could take to 6-8 weeks.

Due to the gap of 3 years between the murders, detective Horgas concluded that the suspect had probably been in prison and searched for someone who got arrested after Carolyn's murder and got released sometime in 1987. Taking this into account, Timothy Spencer became a suspect. Released of prison in 1987, he went to a halfway house in Richmond 2 weeks before the first crime.

On January 20th 1988, Timothy Spencer was arrested. At the time they had little evidence - just the remains of glass from the broken window. After his arrest the police went to his room in the halfway house where they found his pants, ski mask and an army jacket. Lab tests were conducted in a small piece of glass found in the suspect jacket and compared to the shards of the broken window of Susan Tucker's house. Even if both had the same optical proprieties, this evidence was not enough to convict the suspect because the glass was not unique enough to distinguish it from hundreds of other windows. The only physical evidence that could link Timothy Spencer to the crime was DNA.

After his arrest, a sample of his blood was taken and his DNA profile was drawn. Four months after the murder of Susan Tucker, the results were completed – the DNA from the crime scene matched with the Timothy Spencer's DNA. In July 1988, Timothy Spencer trial begun and condemned him to death penalty. In 1989, David Vasquez received a pardon and was released.

This case was the first to convict a suspect of a capital murder in the United States based on DNA profiling [74-76].

1.4.2 - Genetic Background

Every living organism has their own individual DNA and its singularity allows us to distinguish humans from animals or even plants [77]. Most importantly, due to the different combinations of DNA sequences, the study of each "genetic prints" allows an individual identification among humans [77, 78].

DNA can be found in humans cells, namely in blood, hair and sperm – that are also the most commonly samples found in crime scenes [5]. After correctly collected and analysed, it is possible to determine a DNA profile allowing individual identifications [78].

In order to create a genetic profile, a certain amount of genetic material is required. The genetic material can be easily found however, adverse conditions as heat, pH, drought, among others can present negative effects and deteriorate the samples preventing its analysis and posterior DNA profiling [79]. In a crime scenario, after evidences collection, it is necessary to gather samples from the suspects in order to compare both genetic profiles and conclude whether or not the suspects are linked to the crime. Nevertheless, the genetic profile needs to be compared with the victim DNA in order to remove the possibility that the

"suspect" DNA was the one provided by the victim. After processed, the information needs to be interpreted. If both evidence and suspect sample profiling matches, it is required to determine the probability that the profiles correspond with another profile in random population [80].

This way, the use of DNA profiling in criminal investigation can bring benefits to the society by helping to solve crimes, exonerate innocents and assisting in the enforcement of the rule of law.

1.5 - Importance of body fluids identification

Believe only half of what you see and nothing that you hear.

Edgar A. Poe

Serological test allows the detection and identification of body fluids in both native form or as a residue left at a crime scene [15]. Items on which body fluids are thought to be present are submitted to laboratories for serological test and DNA analysis [81]. Before any analysis, it is needed to choose whether the items go through serological test or if they are sent directly for DNA analysis [82]. Indeed, each body fluid requires a different molecular methodology in order to get a more reliable product to conduct a DNA analysis. For example, DNA extraction processes are different for blood and urine. If we conducted the protocol of blood extraction in urine samples it may result in a reduced quality of the isolated DNA, which can compromise the DNA analysis [77, 82].

If fresh blood is fairly easy to identify, bloodstains on grass changes colour quickly, making difficult its identification [82]. Though, this process happens with other biological evidence such as seminal fluid, urine, saliva and so on, when dried or washed. It is why all evidence goes through serological screening first in order to reliably identify the item and ultimately proceed to the molecular process to obtain the most high-quality sample possible. However, samples with trace amounts of DNA goes straight to DNA analysis [79, 82].

Whereas the majority of cases processed involved violent crimes there is been an increase of cases involving DNA to assist in solving property crimes.

Biological evidences are usually left by the burglar. For example, a burglar may injure himself and leave blood at the crime scene or, as an act of vandalism, can relieve himself on the items of the house [48, 83].

As a matter of fact, a complainant's body fluid present on items belonging to a suspect - or vice-versa - holds the most probative value [15]. For example, in cases of sexual assaults, the detection and identification of semen from a sexual assault kit is crucial to support a claim of the same purpose.

In order to be sure, the identification of body fluids through serological analysis is accomplished through presumptive and confirmatory tests. Presumptive tests rely on methodologies that are sensitive and performed quickly, yet they are not specific to the body fluid. Those tests can only indicate if the fluids might be present and do not unequivocally states its presence [77]. On the other hand, confirmatory tests are indeed specific to the body fluid we seek to identify. As presumptive tests, confirmatory testing is sensitive however, it takes a lot more time [77].

1.5.1 - Blood Identification

A comatose teenage female was discovered to be pregnant and the suspected father was no other than a juvenile friend of the victim's brother. In order to elucidate whose the father was, the aborted fetus was submitted along with the victim's and suspect's blood for a paternity test. Blood samples from the victim's brother and father were also submitted. To the general surprise, the suspect was excluded as the father of the fetus, however the results showed that the brother was the one who fathered the fetus.

Adapted from Fisher et al. [84].

The identification of blood can be key-evidence in a wide range of possible situations. Blood identification is essential to many homicide investigations but also in cases involving aggravated assaults, burglary and sexual assaults [85]. The presence of blood can support the complainant's or suspect's version of alleged events establishing culpability or innocence during a criminal proceeding.

Before an extensive blood loss, its detection may not be difficult. However, in some cases where only a very small amount of blood is transferred, its

detection may be very complicated. In cases where the crime scene is been cleaned-up and no blood is visible to the naked eye it is important to rely on serological tests that could allow us to detect and reliably identify blood from clothing, floors, grass or any other surface.

The bright red distinctive colour of blood is derived from haemoglobin. Yet, when it dries, the colour darkens to red-brown and finally to brown, which can difficult its detection and identification.

When an unknown stain is found at a crime scene, the first process we need to use is the so-called presumptive tests. Those tests usually help to elucidate some important questions as: *is it blood?* or *Is it human blood*? [86]. Idealistically, presumptive test must always be supported with confirmatory testing (figure 3).

Till date, there are 3 main presumptive tests for blood: Phenolphthalein, Tetramethylbenzidine and Luminol test [15].

Phenolphthalein test, also known as Kastel Meyer test, is the most frequently used presumptive test for blood [87, 88]. When the chemicals are added, they will react with the haemoglobin present in blood causing the formation of a bright pink colour within 10 to 20 seconds [89]. As its main advantage, this particular technique is very sensitive and highlights stains that are barely visible or even imperceptible to the naked eye [87, 90]. As the others presumptive tests, this particular one does have a major disadvantage – it can easily create false positives once, substance like rust, cooper, among others can also react resulting in a bright pink colour [87].

Tetramethylbenzidine test works the same way as Kastel Meyer, but in this particular case it turns into a blue-green colour instead of a bright pink dye [87, 91]. When compared, tetramethylbenzidine test present a greater level of specificity yet, it does not work as well in diluted blood stains, which is a substantial and important limitation[91].

When blood is suspected to be present but invisible to the naked eye, it would not be reasonable to test the whole item on which the stain may be present. In such occasions, luminol testing is used to indicate nonvisible blood stains [15]. Instead of producing colour change reactions, this chemical presumptive test leads stained areas to emit light [86]. This test is particularly sensitive – even more that the 2 precedent tests previously referred [15]. One disadvantage attached to this test is the same verified with the phenolphthalein test, the occurrence of false positives [67]. In addition to those already named

previously, it also react with bleach and others cleaning fluids which is a problem once it may interfere and create false results on surfaces that have been cleaned [92]. Moreover, when we spray a stain with the chemical, it dilutes the stain. In cases where the stain is already weak, its further dilution may lessen the chances of obtaining a good DNA profile which is another significant drawback to that test.

Nowadays, the Rapid Stain Detection of human blood (RSID™-blood) and the ABAcard® are the most commonly used confirmatory tests in forensic laboratories [87]. Rapid Stain Detection kit enables blood identification through the detection of human glycophorin A, which is present in erythrocytes membrane [73]. Likewise, ABAcard® kit it is very similar to the precedent but detects the presence of human haemoglobin instead of glycophorin A [73, 85].

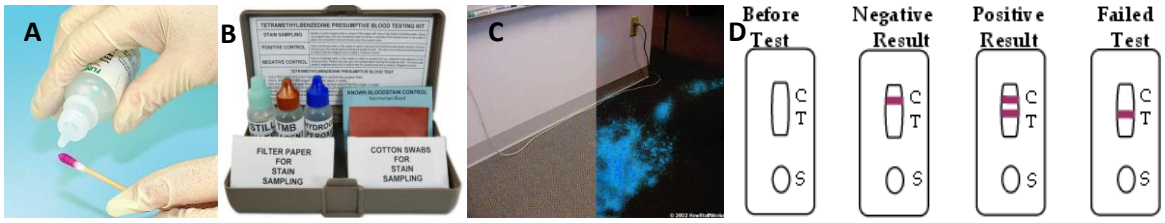


Figure 3 *- Presumptive and confirmatory tests for blood.* Representation of: A- Phenolphthalein test. B- Tetramethylbenzidine test. C- Luminol testing; D- Device used for both ABAcard® and RSID™-blood confirmatory tests.

1.5.2 - Semen Identification

Before the complaint of a sexual assault to a child, a DNA profile was recovered from samples taken from a child's underwear and bedding. His step-father, considered as suspect in this case, argued that the source of DNA was recovered from his skin cells deposited from a casual and frequent contact with the clothes and beddings of the child. For instance, if serological tests were done, in order to elucidate from which tissue the DNA was recovered, and concluded that his DNA was originated from semen and not from skin cells, those results would definitely plays a key-role to support the allegation of sexual assault.

Semen is an extremely important body fluid in cases of sexual assaults [91]. As for example, the identification of semen on vaginal smears swabs or on a victim's clothing may be of value in corroborating the victim's claims.

It is important to underline that not all men produce spermatozoa. Some who have birth defects, have had a vasectomy or as the result of a disease either do not contain or contain very few spermatozoa in their seminal fluid [87]. For that reason, in forensics, 2 major components of semen are particularly important for its identification: seminal fluid and spermatozoa [87].

The most frequently used presumptive test for the detection and identification of seminal fluid relies on the detection of the enzyme acid phosphatase (figure 4) [93]. This enzyme, present in seminal fluid, is also present in others body fluids such as urine, saliva or vaginal secretion yet, in lowest concentrations [87]. The internal concentration of the enzyme allows it be used as a presumptive test for seminal fluid identification, however, its presence in other body fluids leads to a tricky complication: false positives. Acid phosphatase is identified using the brentamine spot test which alters the colour of the tested area into a bright purple colour in a positive case [15, 91]. The absence of colour change not only may indicate that no seminal fluid is present but also may indicate that the level of the enzyme is lower than the detection limit of the test. This is the reason why, when this methodology is used, it is indeed necessary to use additional tests to confirm the presence or absence of seminal fluid [15].

It wouldn't be reasonable to test a large item with the acid phosphatase test to detect nonvisible stains. In order to visualise those stains, an alternate light source is used to pre-screen the item and detect areas that could be processed for acid phosphatase detection (figure 4) [87]. However, not only semen does fluoresce when exited with alternate light but other like saliva or urine does too - hindering its reliability [15].

Items that have been tested presumptively positive can be confirmed by 2 different processes: either by a chemical detection of a specific protein of semen or through a microscopic detection of spermatozoa (figure 4) [48, 87].

Identify spermatozoa microscopically is an undeniable proof that semen was present in the analysed item. Positively tested swabs can be smeared onto a microscope slide and stained by what is commonly called the Christmas tree stain [87]. Composed by two different dyes – nuclear fast red (red colour) and picroindigocarmine (green colour) it enables the visualization of spermatozoa. The head of the spermatozoa will turn red while the tail, if present, will turn green - hence the name of Christmas tree [48, 87]. Nonetheless, seminal fluid belonging to a vasectomise individual does not have spermatozoa therefore, in this situation, microscopic methods do not allows the identification of seminal

fluid [15]. Moreover, the time necessary to microscopically analyse the items is considerable - representing an undeniable drawback when compared to the protein confirmation method.

The protein confirmation method can be done through the Rapid Stain Detection (RSID™-semen). Rapid Stain Detection kit enables semen identification through the detection of prostate-specific antigens (PSA) and when tested positive, serves as an undeniable confirmatory test for semen [87].

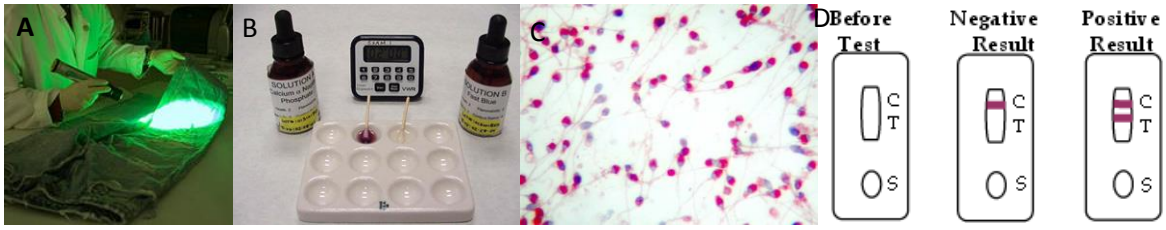


Figure 4 *- Presumptive and confirmatory tests for semen.* Representation of: A- alternate light source test; B- Acid phosphatase test; C- Christmas tree stain; D- RSID™-semen confirmatory test.

1.5.3 - Saliva Identification

An executive in a major company received threat letters and suspected one of his employees who also had an affair with his wife. Though, no arrest or search warrant was up due to the lack of evidence. Later, the chief of the police received a mailed bomb coupled with a threat letter. The saliva present on both treat letters were subjected to a DNA typing and finally compared to the saliva residue found in love letters that the suspect sent to the executive's wife. The DNA types matched and were enough for a search warrant for a sample of blood of the suspect. After confirmation, the suspect was charged with attempted of murder.

Adapted from Fisher et al.[84].

Secreted by the parotid, sublingual, submandibular salivary glands and the mucous glands of the oral cavity, saliva is responsible to keep the mucous membrane moist. It is also responsible for salivary digestion of carbohydrates once the saliva principal component, α-amylase, convert starch into maltose [94].

The transfer of such material can be resulting of direct contact as in food when eating, cigarette butts, straws, drink vessels, envelopes, stamps, bite marks or in cases of oral sex assaults. On the other hand, saliva gathered from an object

as a telephone – mouth piece - result of projected biological material, is a good example of indirect transfer [77].

Currently, saliva screening is mainly done through presumptive tests (figure 6) [95]. Alternate light can be used as a tool for saliva identification, usually in cases where biological fluid are searched in clothing [96]. When excited with alternate light, saliva fluorescence highlights its presence among other biologic samples [96, 97]. However, there is a pretty obvious limitation to that test. Just like saliva, seminal fluid also responds to alternate light resulting in fluorescence (figure 5) [87]. To that point, if the laboratory analyst knows where to look, the problem dimension can be reduced - increasing the probability that the fluorescent stain is connected with saliva. Nonetheless, if it goes the other way and the analyst does not know what he is looking for, he will face a major problem – he will not be able to identify any saliva stains. Due to the fact that many other fluids or even substances can fluorescent with alternate light, this presumptive test is used only to identify areas for additional examinations through other screening tests.

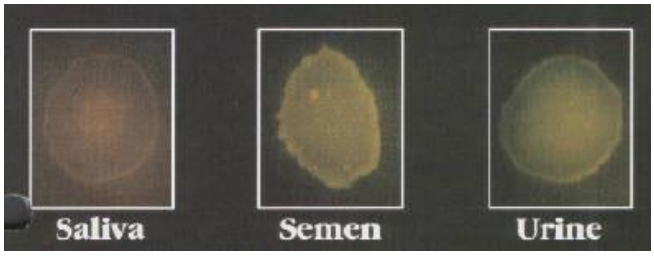


Figure 5- Stains detected by alternate light source – this presumptive test allows the detection of saliva however, other body-fluids do also shows.

Another set of presumptive test for saliva and currently the most widely used is based on amylase detection which is an enzyme that is found at high levels in saliva. The Phadebas® test is used in forensics to identify stains containing amylase and it is performed using paper impregnated with a reagent that dissolves into the paper releasing a blue dye when contacting with amylase [98, 99]. As any other presumptive test, there is some serious limitation to the methodology – Phadebas® test does not differentiate human saliva from other species and, as it was referred before, amylase can be found in other fluids other than saliva and thus this test can identify stains that are not saliva. In order to sidetrack the problem related with species, another presumptive test called Rapid Stain Detection for saliva (RSID™-saliva) was released [100]. RSID™ test, unlike Phadebas®, only recognize human α-amylase [101].

To our knowledge, there is no confirmatory test for saliva identification. However, some laboratories choose to run all 3 tests in order to increase the probability to reliably identify human saliva.

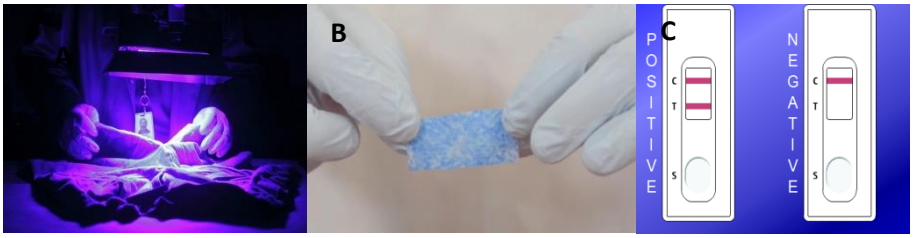


Figure 6 *- Presumptive and confirmatory tests for saliva.* Representation of: A- alternate light source test; B- Phadebas® test; C- RSID™-saliva.

1.5.4 - Urine Identification

Two young brothers accused their neighbour of child molestation, claiming that he gave them pills in order to make them dozy. Blood and urine of both kids were collected for a toxicological screening. Before negative results, the children confessed that they made-up the entire story because they did not like their neighbour.

Adapted from Fisher et al. [84].

The urinating act is commonly found as an act of vandalism and, when gathered, urine can be a source of trace DNA [48]. However, this body fluid is found to be particularly good for drug screening, especially drugs of abuse once nearly all drugs are eliminated in it [48, 102].

There are several simple and fast presumptive tests for urine. The oldest test is undeniably related with its characteristic colour and odour [82]. A simple heating of the samples can emphasise the odour and help with the identification process [82].

The easiest presumptive test is a chemical test named Jaffe colour test. This test is based on the detection of creatinine through the formation of creatinine picrate. A drop of water extracted from the putative stain is mixed with a drop of saturated picric acid followed by a drop of 5% NaOH. The apparition of a bright orange colour indicates the presence of creatinine [82, 103].

Another presumptive test relies on the detection of the major component of dried urine - urea. In the presence of the enzyme urease, urea is cleaved and

formed ammonia which can be easily detected by bromothymol blue. This mapping process uses large sheets of dampened filter paper that allows urine stains to be located when the paper turns bright blue [104]. Due to the limitations associated with those presumptive tests, it is essential to develop more sensitive and specific methodologies.

1.6 - Genetic approach – a broad confirmatory test?

Experience has shown, and a true philosophy will always show, that a vast, perhaps the larger, portion of truth arises from the seemingly irrelevant.

Edgar A. Poe

As previously stated, from a forensic point of view, body-fluids identification are crucial for evidential value but also to guarantee an accurate handling of samples, culminating in a reliable DNA profiling. As it is evidenced, there are a lot of presumptive tests available however, confirmatory tests are way scarcer or simply inexistent. If the reliability of results depends primarily from a correct identification of a certain body fluid, can we really relies ourselves on presumptive tests?

Fact is, for some body fluids like menstrual blood, there is no confirmatory test drawn-up till date. Forensically speaking, there is no shadow of a doubt that menstrual blood is important. If we focus on a sexual crime complain, the presence of blood or menstrual blood in the victim's underwear do not have the same impact. Quite the contrary, a correct identification of the stain could be considered as a major evidence to blame or exonerate the suspect.

Currently, we stand in a molecular epoch where everything seems to find its explication and resolve through a DNA approach. Following this line of thought, it would be reasonable to believe that the ultimate confirmatory test would be based in our genetic background.

If DNA profiling allows the identification of individuals through their respective DNA signatures, it does not identify the type and source of the evidence. Its lack of distinctive signature for each type of body tissue withdraw DNA profiling as a possible candidate as a confirmatory test.

Looking closely at the central dogma of molecular biology, DNA is transcribed into RNA which is translated in protein. Interestingly, RNA does have a specific pattern related to the tissue source. Facing that, is it reasonable to believe that RNA could be a key-factor to answer the dilemma?

1.6.1 - Potential of RNA

Different cell types present in body fluids have different functions and thus, they require a different cluster of functional proteins [105, 106]. Once messenger ribonucleic acid (mRNA) is responsible for the translation of DNA genetic code into proteins, it is logical to consider that different cell types have also a different mRNA pattern expression.

Advantages linked to mRNA profiling are undeniable. It has a great specificity, for example, for any tissue we choose, we are able to find mRNAs that are tissue specific [107]. It is also possible to simultaneously analyse a multitude of markers through common assays. Decreased sample consumption, co-extraction of both DNA and RNA and the automation of the laboratorial procedures are a serious improvement that enhances this methodology [108].

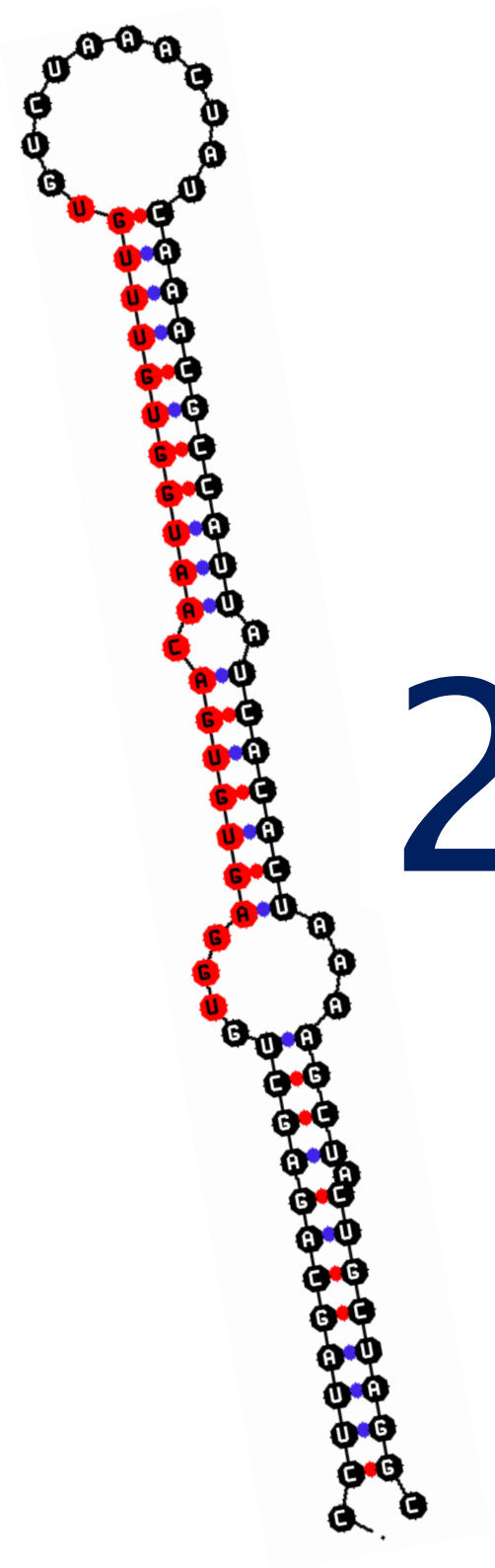
Some important results were published from precedent reports as it is shown on table 1.

Table 1 *– overview of some mRNA published as biomarkers for body fluids.*

	mRNA	REFERENCES
BLOOD	beta-spectrin (sptb)	[109]
	porphobilinogen deaminase (pbgd)	[109]
	aminolevulinate synthase (alas2)	[110]
SALIVA	statherin (stath)	[107, 109, 110]
	histatin 3 (htn3)	[107, 109, 110]
	mucin 7 (muc7)	[110]
SEMEN	protamine 1 (prm1)	[109, 110]
	protamine 2 (prm2)	[107, 109, 110]
VAGINAL SECRETIONS	human beta-defensin 1 (hbd-1)	[109, 110]
	mucin 4 (muc4)	[107, 109, 110]
	keratin 16 (krt16)	[107]
MENSTRUAL BLOOD	matrix metalloproteinase (mmp) 7, 10, 11	[107, 110]

Despite the accomplishment of mRNA profiling, mRNA susceptibility to degradation by physical or chemical factors has always been problematic [111].

The use of RNA presents itself with great potential. Theoretically, mRNA seems to be reliable as a confirmatory test however, its susceptibility is a major impediment. That is why forensic researchers decided to look towards the most studied RNA structure in the past few years: miRNA.



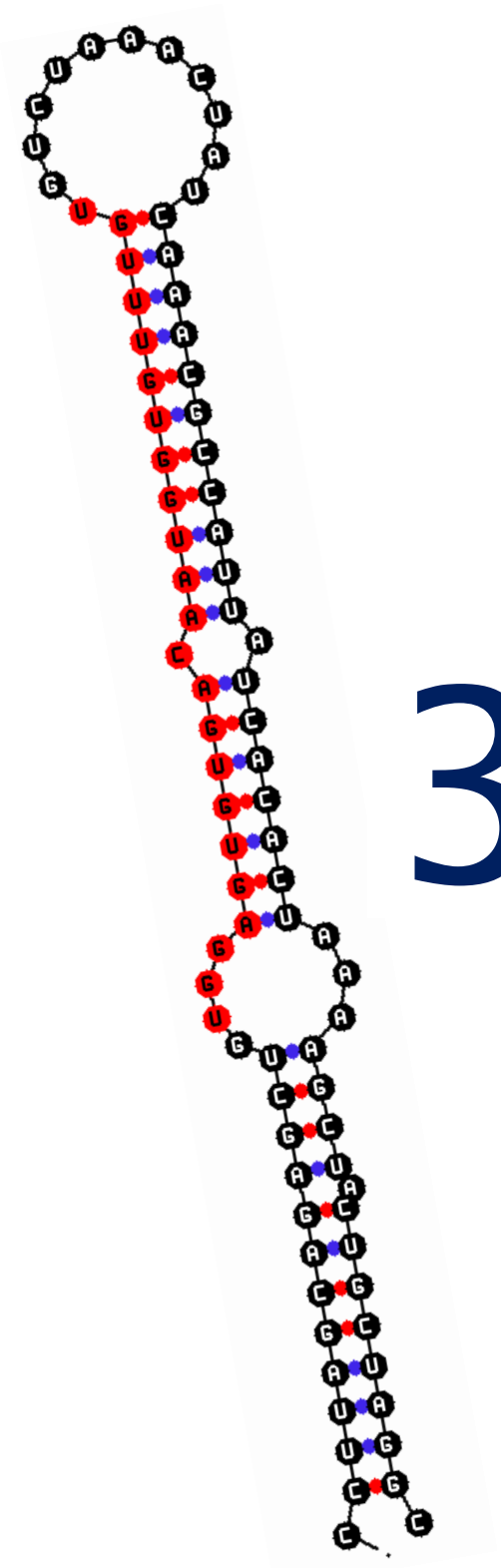
2 Aims

2 - Aims

The aim of the present work was to study miRNA profiles that could be used for the positive identification of some body fluids commonly encountered in criminal investigations.

The first aim consists to a systematic review of all information available till date about the use of miRNAs as a possible biomarker for body-fluids identification. We also acknowledge possible variables that could undermine its potential as a confirmatory test.

The second aim was to determine the relative expression of miRNAs in 2 forensically important body fluid – blood and urine - and extent the study to acknowledge the importance of the quality of the sample and its reflection in miRNA profiling.



3 Material and Methods

3 - Material and methods

Chapter 1 – Systematic Review:

"Forensic miRNA: Potential biomarker for body fluids?"

In forensic investigation, body fluids represent an important support to professionals when detected, collected and correctly identified. Through many years, various approaches were used, namely serology-based methodologies however, their lack of sensitivity and specificity became difficult to set aside. In order to sidetrack the problem, miRNA profiling surged with a real potential to be used to identify evidences like urine, blood, menstrual blood, saliva, semen and vaginal secretions. MiRNAs are small RNA structures with 20-25nt in length that make them less prone to degradation processes when compared to mRNA which is extremely important once, in a crime scene, biological evidences might be exposed to several unfavorable environmental factors. Recently, published studies were able to identify some specific miRNA, however their results were not always reproducible by others which can possibly be the reflection of different workflow strategies for their profiling studies. Given the current blast of interest in miRNAs, it is important to acknowledge potential limitations of miRNA profiling, yet, the lack of such studies are evident. This review pretends to gather all the information to date and assessed a multitude of factors that have a potential aptitude to discrediting miRNA profiling, such as: methodological approaches, environmental factors, physiological conditions, gender, pathologies and samples storage. It can be asserted that much has yet to be made, but we pretend to highlight a potential answer for the ultimate question: Can miRNA profiling be used as *the f*orensic biomarker for body fluids identification?

Chapter 2 – miRNA profiling:

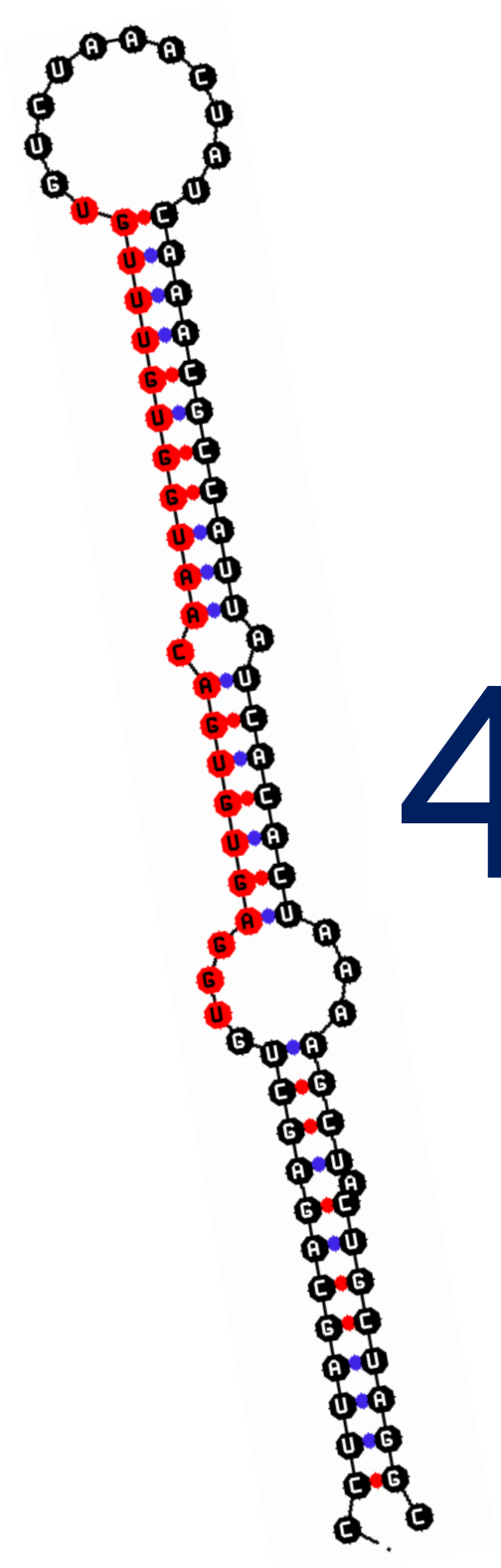
"Circulating miRNA signatures for blood and urine identification".

In this report, we choose to test the presence and expression levels of four miRNAs in both blood and urine samples obtained from 50 healthy individuals from Portugal and study their behavior within those body fluids.

We conducted a miRNA expression profiling study of 50 healthy adult individuals (34% males and 66% females). Our group of study was composed by Caucasian individuals with a mean age of 42, 7 years old, with no major pathological condition. Peripheral venous blood and urine were collected from each subjects following the obtainment of a written informed consent from all subjects. After samples collection, the samples were processed, miRNA extracted and a cDNA library was created.

Afterwards, we proceed to get a relative quantification of our four miRNAs selected - miR-127, miR-221, miR-222 and RNU48 - through real-time PCR.

Statistical analysis was carried out by the computer software IBM®SPSS®Statistics (Version 22.0). In order to assess any statistical alterations in our normalized miRNAs expression essay we used $2^{-\Delta\Delta_{Ct}}$ method and Student's t test.



4 Chapter 1

4 - Chapter 1

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Review

Forensic miRNA: Potential biomarker for body fluids?

Sarah S. Silva[a, b, 1], Cátia Lopes[a, 1], A.L. Teixeira[b], M.J Carneiro de Sousa[a, c], R. Medeiros[a, b]

Show more

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Abstract

In forensic investigation, body fluids represent an important support to professionals when detected, collected and correctly identified. Through many years, various approaches were used, namely serology-based methodologies however, their lack of sensitivity and specificity became difficult to set aside. In order to sidetrack the problem, miRNA profiling surged with a real potential to be used to identify evidences like urine, blood, menstrual blood, saliva, semen and vaginal secretions. MiRNAs are small RNA structures with 20-25nt whose proprieties makes them less prone to degradation processes when compared to mRNA which is extremely important once, in a crime scene, biological evidences might be exposed to several unfavorable environmental factors. Recently, published studies were able to identify some specific miRNAs, however their results were not always reproducible by others which can possibly be the reflection of different workflow strategies for their profiling studies. Given the current blast of interest in miRNAs, it is important to acknowledge potential limitations of miRNA profiling, yet, the lack of such studies are evident. This review pretends to gather all the information to date and assessed a multitude of factors that have a potential aptitude to discrediting miRNA profiling, such as: methodological approaches, environmental factors, physiological conditions, gender, pathologies and samples storage. It can be asserted that much has yet to be made, but we pretend to highlight a potential answer for the ultimate

question: Can miRNA profiling be used as *the f*orensic biomarker for body fluids identification?

Keywords: Forensic science, Forensic serology, body fluids, miRNA profiling, biological biomarkers.

1- Introduction

MiRNAs are small non-coding RNAs with approximately 22 nucleotides of length that seems to regulate a major part of human genes when combined with the RNA-induced silencing complex [1-4]. When this happens, miRNAs controls gene regulation by degradation of the mRNA through cleavage or by preventing protein synthesis [5]. Well conserved in eukaryotic organisms, they are involved in several cellular processes such as apoptosis, development, differentiation and proliferation [6-10]. In recent years, numerous studies showed that miRNAs profiling had a significant role as disease biomarkers, as a powerful tool to understand gene regulation mechanisms such as development mechanisms and gene regulatory networks; and also, due to its tissue specific pattern, in forensic sciences allowing the identification of body fluids [11, 12].

Ambros and co-workers identified the first miRNA, miR-lin-4, in 1993 [13] . They observed that this miRNA was responsible for the timing of development events of *C. elegans* larvar stages, they also discovered that lin-4 was not able to code a protein, but instead encodes what they called small RNAs [13]. Later, a second miRNA, let-7, was described by Pasquinelli et al. and was confirmed its presence in human tissue among different species. This observation demonstrated that these miRNAs were conserved through lineages, making that way, and for the first time, an allusion of an extended phenomenon [14].

If DNA profiling allows the identification of individuals through their respective DNA signatures, it does not identify the type and source of the evidence. Based on the theory that each type of body tissue has a distinctive RNA signature, mRNA profiling surge as an advantageous procedure to identify relevant human body fluids. Despite the achievement of mRNA profiling, the mRNA susceptibility to degradation by physical or chemical factors has always been problematic [15]. In 2009, miRNA profiling to forensic field was introduced by Hanson et al., revealing the ability of miRNAs to identify different body fluids

through miRNA signatures [16]. For example, when a certain miRNA is specific to a human tissue and cannot be found in another sample or when its concentration is significantly elevated and low in other samples, those characteristic profiles of miRNAs may allow the identification of human body fluids such as blood, menstrual blood, semen, saliva and vaginal secretions from others human tissues [16-21]. Argonaute proteins are catalytic component of the RISC complex, responsible for the biological process called RNA interfering [1, 2, 4]. Their tight relationship with miRNA makes them much more stable to degradation processes when compared with mRNA culminating in a superior discriminatory potential, especially in challenging conditions [22, 23].

Through this review we pretended to gather all information available till date about the use of miRNA as a possible biomarker for body-fluids identification. But also, acknowledge possible variables that could undermine its potential as a biomarker.

2- miRNA as potential body fluid biomarkers

Body fluids constitute a beneficial assistance for forensic pathologists and researchers to present means of identifying the perpetrator of a crime and describe how an individual died or suffered through an assault. After being detected and recovered, biological traces need to be identified but some biological stains are difficult to undoubtedly identify, for example, venous blood versus menstrual blood. Through the years, many types of approaches such as chemical tests, immunological tests, microscopy and spectroscopic methods have been used to identify body fluids, however, some of them, like luminol for blood, are presumptive [24]. Henceforth, the potential of miRNA as a biomarker is being studied by means of a molecular genetics-based approach but can miRNA be considered a good biomarker for biological fluids? Idealistically, biomarkers should be able to fulfill a quite imperative number of characteristics [25]. It needs to be available to analyze through non-invasive methods; have a long half-life in samples; be unalterable by physical or chemical factors; it should be specific to a tissue; but most of all, it should be a fast, simple, accurate, reproducible and an economical method [25]. If Hanson and co-workers were the first team to introduce miRNA to body fluids identification in forensic field, soon enough other authors directed their work toward miRNA profiling. Figure 1 displays an overview of all miRNA that different authors considered as possible body-fluid biomarkers.

In this figure it is emphasized the fact that almost all the studies performed had different outcomes and only few miRNAs are confirmed by two or more reports. Studies performed by Hanson, Zubakov, Wang et al. seem to point that miR-16 might be venous blood-specific, and consequently, a potential biomarker for blood stains [16, 17, 20]. Confirmed by three different studies, miR-205 was recognized as a good biomarker for saliva but Wang and colleagues concluded otherwise, sustaining that miR-205 may be epithelium specific and not saliva specific [20]. As a common act of vandalism, the urinating act can be a source of trace DNA but also particularly good for drug screening, especially drugs of abuse [26, 27]. Therefore, it is interesting to underline that no miRNA was detected as potential biomarker for urine, which is an important human fluid in forensics [18]. The lack of homogeneity and the non-reproducibility of results from different groups can be the outcome of dissimilar approaches to reach the one and same goal, highlighting the necessity of standardization for miRNA biomarker assessment.

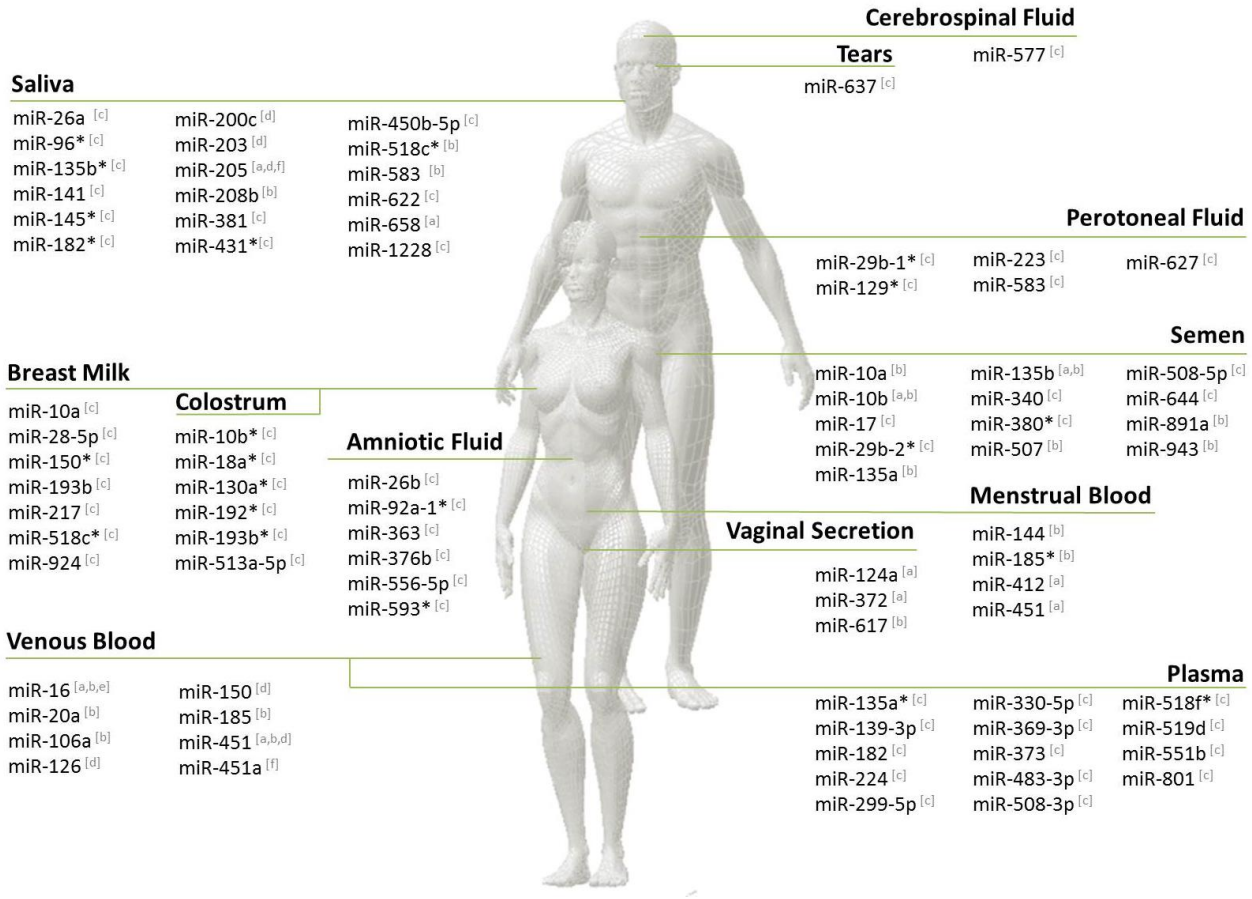

Figure 1 *- overview of all miRNA considered as possible body fluids biomarkers by [a] Hanson et al. [16]; [b] Zubakov et al. [17]; [c] Weber et al.[18]; [d] Courts et al. [19]; [e] Wang et al.[20]; and [f] Omelia et al.[21]*

2.1 – Samples management and storing

The selection of adequate sample, sample processing and RNA extraction are crucial steps for miRNA profiling. The inclusion of degraded RNA in an experiment may result in the inability to detect and quantify specifics miRNA and enable the possibility to get reliable results [28]. However, it is now possible to extract high-quality miRNA from fresh tissues, cell lines, plasma, and serum among other body fluids to guarantee the quality and quantity of the sample, being both of them parameters that have significant impact on the result [18, 29].

Collecting samples is a very important stage to the entire profiling process. When collecting a sample, it might get contaminated with others than the pretended evidence – as ground soil, microorganisms, etc. It is common sense to believe that inadequate handling of the evidence can prevent or confuse the interpretation of the results. Another issue that needs to be raised is the effect of time exposure of the evidence to external factors until its collection and posterior extraction; we also have to consider the post-mortem interval. To our knowledge, there are no published studies that considered their potential effect on miRNAs profiling, nonetheless, we thought important to highlight those interrogations.

Nowadays, dried serum spots are commonly used for body fluid storage, namely blood. Can miRNAs be recovered in good conditions from dried samples? Patnaik et al. published an essay considering miRNA preservation in dried serum blots and they concluded that miRNA preservation was indeed reliable for a posterior profiling. However, they noticed that incomplete drying of blots before storing was prejudicial for its preservation [30].

Acknowledging that criminal investigation can take several years to resolve, can prior extracted miRNA remain in good conditions after mid-term / long-term storage? A very few studies worked to highlight this question with forensic relevant body fluids, though some work was made for human serum [31]. According to the study, four miRNAs were considered to evaluate the effect of storage in human serum at –80ºC (with and without thaw), –20ºC (with and without thaw) and at room temperature. Interestingly, mir-451 was one of the four miRNA considered and, as showed in figure1, it is also one regarded in venous blood. The authors concluded that no significant difference in miRNA levels was verified between –80ºC and –20ºC in short-term storage (10 days). However, when stored at roomed temperature, miRNA levels were drastically decreased, yet still detectable. Moreover, repeated freeze and thaw cycle also

significantly decrease miRNA levels compared with continuous storing, thought that result seems to be inconsistent with other authors research work results [32-34]. For mid-term storage (<20 month) no major differences on miRNA levels were observed between -80ºC and -20ºC, nonetheless some individual miRNA were seriously affected by those conditions. Finally, they explored the effect caused by long-term storage at -20ºC and observed a slightly decrease within the range of 2-4 years; after 6 years of storage a significant decrease of miRNA levels was perceived that only accentuates in the course of time [31]. In summary, miRNA seems to be almost unaffected by freezing temperatures (-20ºC) for at least 2-4 years in human serum, however it is indeed required a similar research work for others human body fluids as they may not respond the same way to such low temperature storage.

2.2 – MiRNA profiling – methodologies

MiRNAs proprieties bring a challenging game to its profiling, with more or less than 22 nucleotides in length (the size of a traditional primer) the traditional primer binding is impossible. In order to sidetrack the problem, the elaboration of a smaller primer is required, demanding a lower melting temperature and ultimately affecting the efficiency of the PCR. Furthermore, the short length and discrepancy in GC contents leads to a wide variance of melting temperatures resulting in another challenge to its reliable profiling.

Nowadays, a large cluster of methodologies has the capacity to be used for miRNA profiling. Methodologies like Northern Blotting using locked nucleic acid, RNase protection essays and in situ hybridization methodologies are known for miRNAs detection [35-37] . However, we decided to highlight the three major methods that are being currently used: microarray (hybridization-based method), qRT-PCR (quantitative reverse transcription PCR) and RNA sequencing. Interestingly, the groups taken in considerations for their work in miRNA profiling for body fluids identification used different combinations of methods. Could those combinations be responsible for the heterogeneity of results showed in figure 1? This hypothesis was suggested by Zubakov et al. when they tried to replicate the results obtained by Hanson and colleagues – for vaginal secretions, menstrual blood and saliva – and failed [17].

The concept of DNA microarray technology resides on the ability of a single strand of DNA (probe) to bind to a complementary strand of DNA (identified as target). The targets miRNA are initially reversed into cDNA and

fluorescently labeled. Once the targets successfully hybridized with the probes (known sequences) the microarrays are scanned to detect the fluorescence of the DNA probes, which can be measured using a fluorescent scanner [38]. Microarray technology has, as its main advantages, the ability to study the expression of thousands of genes or their RNA products at once involving a fairly low cost price. However, despite its high-throughput, the lack of specificity and sensitivity compared to other methods such as qRT-PCR or RNA sequencing is considered as a real disadvantage that can tamper the results. Another limitation resides on the large amount of sample required to do the experiment, which is a major problem with forensic samples. Moreover, microarray only provides results about the genes that are included in the array itself preventing the identification of novel miRNAs and unlike qRT-PCR it cannot be used for absolute quantification.

RNA sequencing (RNA-seq) is established as a high-throughput sequencing method and one of the major approaches to miRNA expression profiling. RNA-seq technology begins with a reverse transcription of the miRNA to a cDNA library [39]. Once the library is created, adaptors are applied to one or both limits of the sequences enabling a massive sequencing of the cDNA library [40]. As a high-throughput next-generation sequencing platform, RNA-seq is the only method able to identify both known and most importantly, novel miRNAs but also, differentiates miRNAs that are very similar, namely, distinguish miRNA that diverge by only one nucleotide resulting as a real advantage when compared to microarray and qRT-PCR technologies. Just like microarrays, RNA-seq cannot be used for absolute quantification but also presents as a major disadvantage the necessity of an important support of bioinformatics tools which presents relevant challenges through the process. Moreover, the cost associated with RNA-seq *per* sample is still high although it has been decreasing over these last years.

Quantitative reverse transcription PCR (qRT-PCR) relies on reverse transcription of miRNA to cDNA, followed by qPCR and posterior real-time monitoring of the resulting products [41]. qRT-PCR is a very sensitive and specific (especially with TaqMan®–based detection) compared to the other two precedent approaches and can be used for absolute quantification. In forensic cases, quantity of genetic material collected can be little and therefore, it is important to use a methodology that requires a fairly low amount of RNA. qRT-PCR beyond being a well-established method, it also requires a low amount of RNA and has a fairly low cost per sample. On the other hand, it also present a significant disadvantage, it cannot identify novel miRNAs.

Hanson, Zubakov and respective co-workers used qRT-PCR for their experiment. However, Zubakov et al. failed to replicate some of the results reported by Hanson and co-workers [17]. Even using the same platform, two different methodologies were performed: SYBR® Green and TaqMan®. SYBR® Green detects PCR products by binding, nonspecifically, to double-stranded DNA, which can generate false positive results [42]. On the other hand, a TaqMan® approach relies on a specific hybridization between probe and target, required to generate fluorescent signal [42]. The incorporation of target and probe leads to an increase of the sensitivity and specificity of the qRT-PCR platform. Those different methodologies are considered by Zubakov et al. as one possible reason for the discrepancy of results. Moreover, when compared the workflow of the authors considered for this review, not only the methodologies used were different but also their choice of the normalization gene. Used to remove disparities across samples, and despite its crucial importance to obtain accurate results, there are no consensus normalizations for either of the methods reviewed [43]. This reinforces the necessity to define a concrete workflow for miRNA profiling and standardize each method (fig. 2).

2.3 - Gender and miRNAs

In order to elucidate if miRNAs can or not be considered as biomarkers for body fluid identifications, we gather the information in published reports that involves miRNAs presented by authors as biomarkers for human body fluids, and show their different expression levels when influenced by both biotic and abiotic factors. In this section, miRNAs profiles that differ with gender will be the main focus.

The research work done by Duttagupta et al. highlighted miRNAs that showed to be gender specific in normal male and female population. A microarray analysis performed in human plasma, emphasize a subset of four miRNAs (miR-548-3p, miR-1323, miR-940 and miR-1292) that were found to be up regulated in females (63–95%), demonstrating its potential to distinguish gender. Interestingly, none of these miRNAs were significantly down-regulated [44]. Those results would be expected if the concerned miRNAs were sex tissue specific, namely mir-940. As cervix specific, it is logical that mir-940 is found up-regulated in women plasma when compared to male samples. However, the other three miRNAs were not female sex tissue related, yet, they also were up-regulated. In 2012, Wang et al. showed that mir-130b and mir-18b had slightly higher

concentrations in male serum samples compared to females [45]. Though, we should note that the authors were not able to exclude the possibility that both miRNAs may be associated with gender-association functions [45]. Furthermore, another research was drawn up in 2013 and studied the association of circulating serum miRNAs with the metabolic syndrome (MetS) and related it to gender. They attested a significant change in the miRNA profile, notably for two miRNAs, let-7g and miR-221, which were overexpressed in the serum of individuals with MetS ($P = 0.004$ and $P=0.010$, respectively) and more prominent in women [46].

Idealistic biomarkers should not be expected to change by any means, however any discrepancy to that rule may underline its role as a biomarker. In a forensic point of view, it would be reasonable to question whether those gender-related differences in miRNA levels may occur in others biological fluids because, if non gender-tissue specific profiles of miRNAs changes, those miRNAs may not be suitable as biomarkers (table 1).

2.4 - Pregnancy and miRNAs

Recent reports have shown that a large number of miRNAs are expressed in human placenta. These miRNAs, produced by human trophoblast cells, can be secreted into maternal serum or plasma through an exosome-mediated pathway [47-50]. There is why we can question ourselves whether or not pregnancy affects miRNAs profiling. In this section, we show that pregnancy indeed leads to different expression level of some miRNAs, especially in those pointed out as possible biomarkers for body fluids.

Chim et al. searched for placental miRNAs in maternal plasma and detected four miRNAs (miR-141, miR-149, miR-299-5p, and miR-135b) at higher concentrations in the maternal plasma before delivery when compared to after delivery and concluded that these miRNAs were placental-specific. The plasma concentration of miR-141 increased with gestational age showing the highest concentration in the third trimester [48]. Defined by different authors as saliva-specific, miR-141 was reported by Chim et al. as placental-specific in maternal plasma [48]. Therefore, further studies are needed to confirm these results. Interestingly, miR-299-5p, showed as plasma specific works also as placental-specific when up-regulated.

By studying a panel of 723 human miRNAs, *Miura* et al. found that 24 miRNAs showed significantly decreased concentrations in maternal plasma after

the termination of delivery. 13 of the 24 genes encoding pregnancy-associated miRNAs were expressed only in placenta and the remaining 11 miRNAs were produced predominantly in placenta tissue [49]. These miRNAs were considered pregnancy associated and almost all are located in chromosome 19, known to contain a large cluster (C19MC) of miRNAs genes and other genomic elements involved in placental growth and development [51, 52]. Other placental miRNAs (miR-516-5p, miR-517*, miR-518b, miR-520a*, miR-520h, miR-525 and miR-526a) were detected in maternal plasma by Kotlabova et al. and recognized as pregnancy associated miRNAs [53]. Gilad et al. compared serum miRNAs levels in pregnant and non-pregnant women and observed that three of them were highly expressed during pregnancy (miR-526a, miR-527 and miR-520d-5p) [50, 51].

Many miRNAs have also been detected in trophoblast cells, a heterogeneous group of fetal cells formed during the first stage of pregnancy [54, 55]. Morales-Prieto et al. analyzed these cells in different stages of pregnancy (first and third trimester) and showed that the level of C19MC miRNAs increases significantly from first to third trimester, while C14MC miRNAs levels have the opposite pattern. Also, miR-371-3 cluster had a small increase with advance of gestational age. These clusters have several miRNAs that are considered placental-specific [56]. Nevertheless, more studies are needed to compare these results with non-pregnant women and with other body fluids.

2.5 - Age-related miRNAs

MiRNAs have recently emerged as important regulators of aging and, since then, the number of studies has increased. The first genetically identified miRNAs were *Caenorhabditis elegans lin-4* and *let-7*. These and others miRNAs have been well-studied in this model and seems to show that it regulates lifespan and predicts individual longevity [57-60]. Boehm and Slack found that the overexpression of miRNA *lin-4* results in an extended lifespan of *C. elegans*, whereas the loss of *lin-4* function has an opposite effect [61]. Ibáñez-Ventoso et al. suggested that, since many age-related miRNAs are conserved, their human homologs might be similarly regulated to modulate age-related decline and age-related pathology [57].

By definition, idealistic biomarkers should not be altered by any means. It is why it is indispensable to study miRNA profiles when targeted by both internal and external factors in important forensic body fluids. Only the ones stable enough have the potential to be considered biomarkers. Several studies in mice,

primates and humans compare expression levels of miRNAs in younger and older tissues but there are only a few works about the age-related changes in miRNAs levels in biological fluids.

Li et al. studied the expression of miRNAs in rat liver during aging and observed that miR-34a and miR-93 increase in the middle and old-age rat liver, compared to young rats [62]. Maes et al. also investigated the expression of miRNAs in the same tissue and proved that a gradual increase of miR-669c and miR-709 was observed from the mid-age (18-33 months), whereas miR-93 and miR-214 are increased in extremely old (33 months) mice [63]. The up-regulation of these miRNAs caused a repression of oxidative defense genes by targeting transcription factors important for detoxification and regeneration of the liver, which decreased with age [58, 62, 63]. Other research work done by Li et al. examined the role of miRNA regulation in mouse brain during the normal aging process and their results suggested that, like in the liver, the expression of miR-22, miR-101a, miR-720 and miR-721 increased with aging, starting from mid-adulthood, with this up-regulation being observed since 18 months of age [64]. The majority of the up-regulated miRNAs were predicted to target and correlate in expression with components of the mitochondrial complexes [58, 59, 64]. Other studies were done in mice tissues, like skeletal muscle, by Hamrick, Drummond and respective co-workers [65, 66].

Somel et al. found that few gene expression changes are unique to aging. They measured miRNA expression in the prefrontal cortex of humans and rhesus macaques and detected that, in human, 31% of 373 miRNAs showed significant expression changes with age. These results had good positive correlation between humans and macaques ($P < 0{,}020$) [67]. Recently, it has been established the first miRNA and noncoding RNA expression atlas of the developing human brain with differential expression signatures between fetal, early postnatal and adult brain tissue samples [68]. Various miRNAs have also been shown to be down-regulated in peripheral blood mononuclear cells from old American individuals (miR-103, miR-107, miR-128, miR-130a, miR-155, miR-24, miR-221, miR-496, miR-1538) compared to younger individuals [59]. The loss of miRNA function during the aging process may be due to transcriptional repression, deletion, mutation, epigenetic silencing or aberrant miRNA processing and suggest that miRNAs and their predicted targets have the potential to be diagnostic indicators of age [59]. ElSharawy et al. identified 80 age-related miRNAs in blood samples with 16 miRNAs being up-regulated and 64 (80%) down-

regulated compared to younger individuals. Their results showed that some miRNAs identified previously by Hooten et al. also appeared in this study, such as miR-103, miR-107, miR-24 and miR-130a [69]. Hackl et al. also identified four down-regulated miRNAs that are involved in aging and, three of them, were confirmed by ElSharawy et al. (miR-17, miR-20a and miR-106a) [69, 70]. It is interesting to note that some of these miRNAs are also considered by some authors as body fluids biomarkers (Fig. 1). For example, miR-17 was recognized as a good biomarker for semen and a probable age-related miRNA; miR-130a seems to be colostrum-specific and an aging biomarker; miR-20a and miR-106a, which were found in blood samples, were recognized as venous blood-specific miRNAs but also age-related miRNAs. Recently, other research work was done in body fluids. Hooten et al. provided evidence that, in human serum, miRNAs were differentially expressed with aging. In this study, they found a significant decrease in expression of miR-151a-5p, miR-181a-5p, and miR-1248 and it happened regardless of gender and race, suggesting that they may be good age-related biomarkers. All three miRNAs were predicted to be central mediators of inflammatory pathways and important for organismal development and survival, suggesting that a decreased expression may result in the development of age-related phenotypes and diseases [60]. However, it would be necessary further studies to corroborate all these results obtained so far.

2.6 - Environmental factors

In order to be considered as a confirmatory test in forensic serology, it is important to acknowledge abiotic factors that could alter miRNAs expression levels in human body fluids. The interaction between miRNAs and environmental factor is undeniable. More importantly, miRNAs signatures seem to be induced by some factors as temperature, drought, pH, among others. In this chapter, we scrutinized the still slightly studied topic.

2.6.1 – Temperature

Recently, miRNAs have been linked to environmental stress factors, however, the relationship between miRNA expression and stress responses is just beginning to be explored.

MiRNA expression is modulated by temperature, dehydration, radiation and other factors. In plants, 19% of miRNAs are involved in the response to

temperature stress [71]. Sunkar and Zhu demonstrated that miR-393, miR-397b, miR-402 and miR-319c, in *Arabidopsis thaliana,* were up-regulated by cold (low temperature) whereas miR-389a was down-regulated [72]. The study done by Lee et al. identified miR-156, miR-163, miR-169, miR-172, miR-398 and miR-399 as temperature-responsive miRNAs of Arabidopsis [71]. MiR-393 was also strongly up-regulated by cold in the research work done by Liu et al. but also reported by others in other plants [73, 74]. In orchid *Phalaenopsis*, four low temperature-responsive miRNA families were identified – miR156, miR162, miR528 and miR535 [75]. Though, the expression of miRNAs not only changes with decreasing temperatures. May and colleagues observed that some miRNAs changes their expressions by increasing temperature 3-6ºC and, among all predicted miRNAs, four were increased significantly [76].

Some studies have shown that, due to extreme environmental conditions, the expression of miRNAs is changed in both invertebrates and vertebrates animals. For example, it was evaluated the freeze tolerance in frogs and gastropods and the hibernation in ground squirrels [77-79].

Till date, there are a very few studies toward human body fluids and environmental factors impact on them. There is why we decide to look toward miRNA behavior in plants, invertebrates and vertebrates when conditioned by adverse conditions. However it is important to acknowledge that in plants, miRNAs have some differences functions compared to animals.

In humans, Benson and colleagues studied the variability of miRNA in whole blood, stored until 12 hours at room temperature. They concluded that MiR-16 and miR-223 were not altered by room temperature and established that these miRNAs were stable in whole blood and could be used as plasma-specific miRNAs [80]. However, more studies are needed in order to understand whether human miRNAs are altered by temperature in body fluids.

2.6.2 – Radiation

The expression of specific plant or animal miRNAs has been shown to be altered under abiotic stresses such as radiation. Many studies have been done to prove that these miRNAs play important roles in response to radiation, as UVB stress in plants. The results obtained suggested that each plant has its own special UVB-regulated network [81, 82].

It was explored the expression of miRNAs in mouse peripheral blood and the study concluded that miRNA expression signatures were radiation type-

specific and dose and time-dependent [83]. Furthermore, Dressman et al. showed that radiation-induced gene expression signatures derived from mouse blood can predict the irradiation status of human samples with very good accuracy [84].

In humans, several studies were performed. Kraemer et al. showed that UV irradiation of human primary keratinocytes modulates the expression of several cellular miRNAs, comparing the effect of UVA and UVB irradiation on miRNA expression. In UVA-irradiated cells, 27 miRNAs were differentially expressed compared to non-irradiated cells, whereas in UVB-irradiated cells 28 deregulated miRNAs were identified. These results demonstrate that miRNAs are differentially expressed in response to UVA and UVB and can play an active role in the diagnosis of some cancer diseases [85]. Other study, done by Templin and colleagues, investigated the potential of radiation-induced miRNA expression profiles in peripheral blood cells to provide biomarkers for radiation exposure that could be used in therapeutic or diagnostic settings. The results were obtained immediately before and after four hours of total body irradiation with 1.25 Gy x-rays and they concluded that 45 miRNAs were statistically significantly up-regulated after irradiation. This study suggested that miRNA expression signatures can be used as biomarkers of radiation exposure [86].

Some studies on the influence of radiation exposure in miRNAs were performed in body fluids. Cui and co-workers showed that plasma miRNA expression signatures distinguished mice that received total body irradiation doses of 0.5 Gy, 2 Gy, and 10 Gy with accuracy, sensitivity and specificity above 90%. According to these, they concluded that plasma miRNA profile can be predictive of different levels of radiation exposure [87]. The evolution of miRNA expression subjected to different ranges of radiation may also be indicative for various physiological responses and pathological stages. Jacob and associates compared 600 miRNAs in serum and they observed that miR-150, abundant in lymphocytes, exhibited a dose and time dependent decrease with increase of radiation range and was suggested as a marker indicative of lymphocyte depletion and bone marrow damage [88]. Interestingly, miR-150 was considered as body fluid-specific (venous blood) but also a good biomarker for radiation.

2.1.2 – Other environmental factors

There are other factors that are still slightly studied, but which need to be mentioned as promoters of the differential expression of miRNAs, such as drought or dehydration, pH and salinity.

One of the first studies in this field was done by Sunkar and colleagues. In this study, a pair of samples from *Arabidopsis* (untreated and treated) was assayed for each treatment and total five microRNAs were observed to be regulated by one or more of environmental factors [72]. Liu et al. also studied, in Arabidopsis, the expression of miRNAs to environmental stress factors and concluded that 14 of them were differentially regulated by one or more stress conditions. MiR-168, miR-171 and miR-396 responded to high-salinity, drought and low temperature [73]. The research work done by Zhao et al. compared their results with the study from Liu and observed that three miRNAs (miR-319c, miR-393 and miR-397b) had conserved homologs in rice [89]. MiR-393 and miR-397b were up-regulated by dehydration in both studies. Furthermore, the up-regulation of miR-169g due to dehydration suggested that this miRNA might play an important role in drought stress in rice [73, 89]. Other three studies also observed that miR-393 was up-regulated during drought stress in various plant species [90-92] . As mentioned above, many studies have already been made on plants but studies in animals are lacking. Recently, Wu et al. analyzed the effects of whole body dehydration on miRNA expression in three tissues (liver, kidney and skin) of African clawed frog, *Xenopus laevis*. This study showed a tissue specific mode of miRNA expression during dehydration [93].

There are no developed searches in body fluids subject to dehydration or drought but, from these studies, it is possible to expect that what happens in plants and animal tissues may happen also in body fluids.

2.7 – Pathologies and specific body-fluid miRNAs

A considerable amount of the research work done toward miRNAs is directed to pathological cases. By all means, its "expression variation" seems to pinpoint biological deregulations that trigger several events as invasion, metastasis, proliferation and differentiation in cancer, but also responsible for neurodegenerative disorders [94-99], inflammation processes [23, 30, 35, 36, 39, 100, 101] and infectious diseases [102-105]. Also recognized as oncomirs, miRNAs can act as oncogenes when they are found in higher expression levels, culminating by promoting tumor development; but also as tumor suppressor, when their expression is reduced in cancer cells, preventing tumor development [106, 107].

Once again, if we assume the possibility of miRNAs as biomarkers for body fluid identification, they should be stable regardless different internal conditions

however, upon pathological conditions, miRNAs different expression levels seems to have a key-role importance.

The samples used for the research seems to be confined to tumors cells and plasma/serum, but only few numbers used the ones we approaches for this review. Here, we will reveal some miRNAs with altered levels of expression in cancer. Acute Myeloid Leukemia is a cancer of the myeloid lineage of leukocytes that interferes with the production of normal blood cells and after reviewing the literature, it was found a decreased of miR-150 in human plasma [108]. It is also important to observe that miR-150 was determined by Court and Medea as a venous blood specific miRNA [19]. For bladder cancer, some studies were done in urine samples and reported an up-regulation of miR-96, miR-126 and miR-182 [109, 110]. Weber et al. considered the high concentration in plasma of miR-182 as a biomarker for plasma identification when compared to other body fluid samples [18]. However, if in bladder cancer, miR-182 is up-regulated in urine, can this miRNA be a good biomarker for plasma? Can we really distinguish both samples when miR-182 is up-regulated in patients with bladder cancer? Clearly, more studies are needed to be done toward those problems in order to clarify which miRNAs can be *really* considered as "specific" that is, idealistically not altered by any means. For breast cancer, some miRNAs were decreased in both plasma and serum samples [111-115] but other interesting miRNAs were found increased such as miR-10b, miR-141, miR-200c, miR-203, miR-373, miR-451 and miR-801 among others in plasma [112, 113, 116, 117]. In serum, the literature pinpoint a large amount of miRNAs as up-regulated but it is to mention that miR-10b is founded to be also up-regulated just as miR-299-5p [114, 115, 118-120]. In patients with colorectal cancer, miR-141 was found up-regulated in plasma [121]. In esophageal squamous cell carcinoma, it was showed an increase of miR-10a and miR-223 in serum samples [122]. MiR-106b was found to be up-regulated in plasma while miR-20a was up-regulated in both serum and plasma in patients with gastric cancer [123-125]. Lung cancer is one of the diseases with more associated miRNAs biomarkers in literature to date. In plasma, miR-16, miR-106a and miR-126 were found decreased while miR-182 and miR-451 were found increased [126-129]. Moreover, in serum, miR-16, miR-106a, miR-141, miR-200c and miR-593* were down-regulated whereas miR-10b, miR-182, miR-205 and miR-223 were up-regulated [11, 107, 127, 130, 131]. For prostate cancer, it was showed an up-regulation of miR-26a and miR-141 in serum [32, 132, 133].

The unique profile of miRNAs in cancer and their expressional variation in different types of tumors reveals an additional layer of complexity for body fluid identification through miRNAs. As it was said for the others variables, more studies need to be planned to answer whether miRNA can or not be used as a reliable biomarker for body fluid identification.

Table 1 – Overall of some of the human miRNAs considered for this review.

Variable	Deregulation	Tissue / Body Fluid	miRNAs				References
Gender							
Female	Increase	**Plasma**	miR-940	miR-548-3p	miR-1292	miR-1323	[44]
		Serum	let-7g	miR-221			[46]
Male	Increase	**Serum**	miR-18b	miR-130b			[45]
Pregnancy	Increase	**Plasma**	miR-135b	miR-141	miR-149	miR299-5p	[48, 53]
			miR-516-5p	miR-517*	miR-518b	miR-520a*	
			miR-520h	miR-525	miR-526a		
		Serum	miR-526a	miR-527	miR-520d-5p		[50]
Age	Decrease	**Blood**	miR-17	miR-20a	miR-24	miR-103	[59, 69, 70]
			miR-107	miR106a	miR-128	miR-130a	
			miR-155	miR-221	miR-496	miR-1538	
		Serum	miR-151a-5p	miR-181a-5p	miR-1248		[60]
Radiation	Decrease	**Serum**	miR-150				[88]
Pathology							
Acute M. Leukemia	Decrease	**Plasma**	miR-150				[108]
Bladder Cancer	Increase	**Urine**	miR-96	miR-126	miR-182		[109, 110]
Breast Cancer	Increase	**Plasma**	miR-10b	miR-141	miR-200c	miR-203	[112, 113, 116, 117]
			miR-373	miR-451	miR-801		
		Serum	miR-10b	miR-299-5p			[118-120]
Colorectal Cancer	Increase	**Plasma**	miR-141				[121]
Esophageal Cancer	Increase	**Serum**	miR-10a	miR-223			[122]
Gastric Cancer	Increase	**Plasma**	miR-20a	miR-106b			[123, 124]
		Serum	miR-20a				[125]
Lung Cancer	Decrease	**Plasma**	miR-16	miR-106a	miR-126		[33, 127, 128]
		Serum	miR-16	miR-106a	mi-141	miR-200c	[107, 130, 134]
			miR-593*				
	Increase	**Plasma**	miR-182	miR-451			[33, 128, 129]
		Serum	miR-10b	miR-182	miR-205	miR-223	[11, 131, 135, 136]
Prostate Cancer	Increase	**Serum**	miR-26a	miR-141			[32, 133]

3 - Conclusion and future considerations

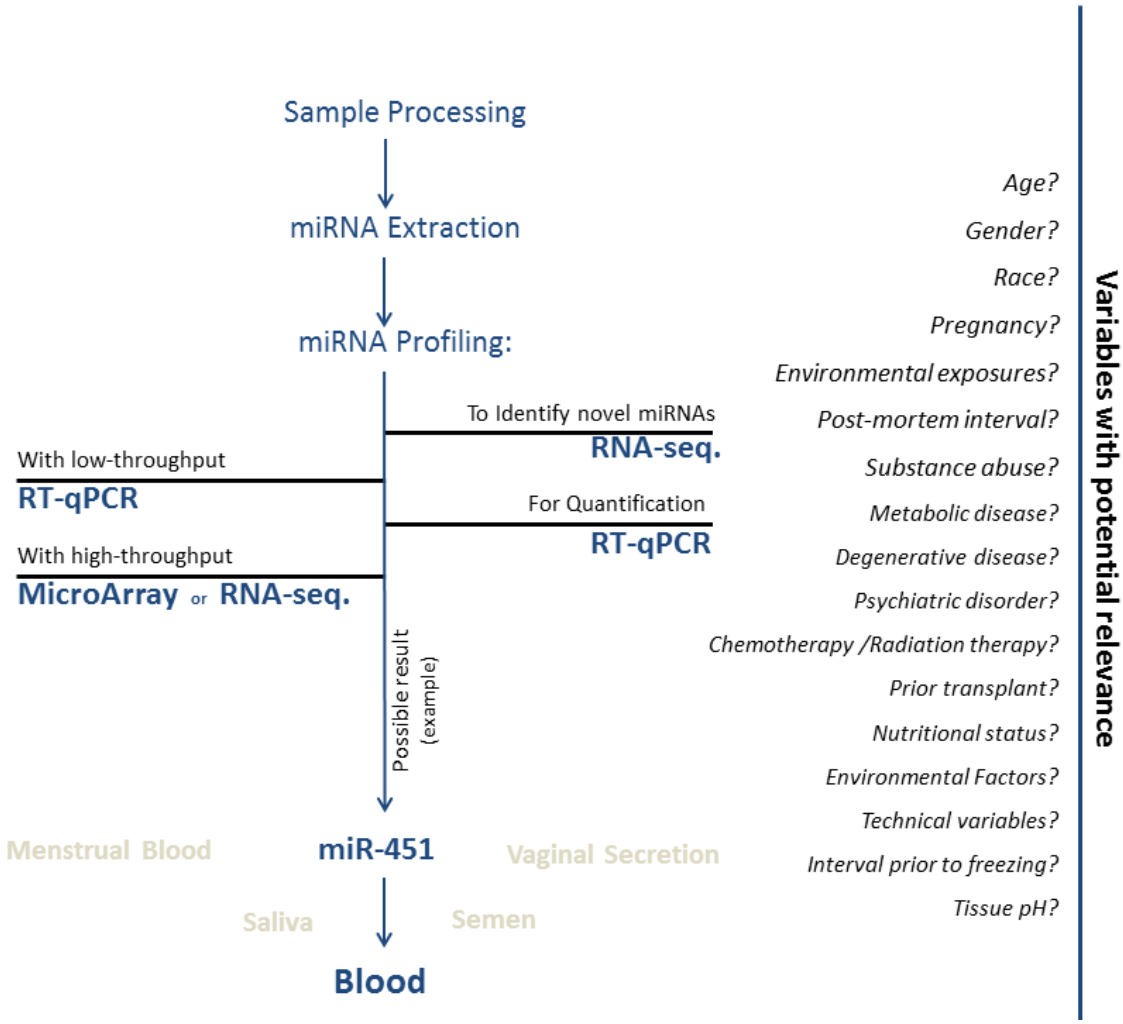

Figure 2 – miRNA profiling workflow for body fluid identification and potentially relevant variables that need to be study.

Studies involving miRNAs are still in its early stages and that is why every factor with potential relevance needs to be studied. The main problem associated with miRNA as to do with the rush to go straight to the main goal without studying those variables. When the scientific community started to talk about miRNA profiling in a forensic purpose, numerous studies surged with what they considered miRNA body fluids-specific. However, when reviewed all the results, it is easy to question whether or not physiological conditions, genetic, clinical, technical and pre-analytical variables have the power to induce other outcomes. Within these review, we tried to elucidate some of those variables assessing parallel studies that mentioned some of their effects. However, mostly of them were not aimed toward biological fluids (at least not on the forensically important

ones) and, as in all biological sciences, the parallelism between results leads to obvious mistakes. That is why, before trying to obtain a profile it is indeed needed to stop and focus on every possible situation to enhance the chances to achieve in reliable and reproducible results.

Here, we started to question problems related with storing conditions and we concluded that they were indeed affected, yet, those result were obtained from serum. In this situation it is reasonable to question how blood, semen, saliva, urine and vaginal secretions samples would be affected by short/long-term storage. We also reviewed the three major methods used to date: microarray, qRT-PCR and RNA sequencing, all of them with their advantages e disadvantages. At this point, there is no convention between laboratories for miRNA profiling despite the fact that it is consensual that different platforms lead to different results. The reflection of that problem can easily be spotted, for example, when some authors tried to reproduce some of other group results and failed, enhancing the necessity to define a concrete workflow for miRNA profiling assessment. Can gender or physiological conditions such as age or pregnancy alter microRNA profiles in body fluids samples? Apparently yes, however it would be necessary to expand the research to other body fluids. Are those alterations also ascertained in other body fluids other than serum and plasma? There is no evidence to date, however it would be very important to acknowledge this possibility. In theory, if I assume that a certain miRNA is in a higher concentration in venous blood and low in menstrual blood, that concentration variation would allow the distinction of those two samples. However, if for some reason miRNA levels were altered, this theory would lose its purpose and undermine miRNAs potential as a biomarker. Today, a large amount of the research with miRNAs regards pathological conditions namely cancer. MiRNAs expression variation reflects biological deregulations and it can be observed within a various amount of human tissue. Unlike the precedent factors, there are more studies that include forensically relevant body fluids such as blood and saliva. However, it would be required to verify those variations in other body fluids.

These data pretended to give an overall look, in a forensic point of view, of miRNA profiling and a multitude of factors that have the potential of altering it. Despite the unquestionable potential of miRNA profiling, the research work done to date has raised a number of interrogations and it would be wise to start *researching* their answer. Our resumed conclusions would be recapitulated in response to two questions: Are miRNAs *the* forensic biomarkers for body fluids

identification? No, at least not today. Can miRNAs be *the* forensic biomarkers for body fluids identification? We would say yes, however there is a lot of work to be done to reliably answer that question.

Conflict of interest

None.

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References

1. Hannon, G.J., *RNA interference.* Nature, 2002. **418**(6894): p. 244-251.
2. Hutvagner, G. and M.J. Simard, *Argonaute proteins: key players in RNA silencing.* Nat Rev Mol Cell Biol, 2008. **9**(1): p. 22-32.
3. Santos, J.I., et al., *Restoring TGFbeta1 pathway-related microRNAs: possible impact in metastatic prostate cancer development.* Tumour Biol, 2014.
4. Varallyay, E. and Z. Havelda, *Unrelated viral suppressors of RNA silencing mediate the control of ARGONAUTE1 level.* Mol Plant Pathol, 2013. **14**(6): p. 567-75.
5. Dias, F., et al., *Renal cell carcinoma development and miRNAs: a possible link to the EGFR pathway.* Pharmacogenomics, 2013. **14**(14): p. 1793-1803.
6. Jovanovic, M. and M.O. Hengartner, *miRNAs and apoptosis: RNAs to die for.* Oncogene, 2006. **25**(46): p. 6176-87.
7. Pauli, A., J.L. Rinn, and A.F. Schier, *Non-coding RNAs as regulators of embryogenesis.* Nat Rev Genet, 2011. **12**(2): p. 136-49.
8. Tay, Y., et al., *MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation.* Nature, 2008. **455**(7216): p. 1124-8.
9. Esteller, M., *Non-coding RNAs in human disease.* Nat Rev Genet, 2011. **12**(12): p. 861-74.
10. Bartel, D.P., *MicroRNAs: Genomics, Biogenesis, Mechanism, and Function.* Cell, 2004. **116**(2): p. 281-297.
11. Chen, X., et al., *Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases.* Cell Res, 2008. **18**(10): p. 997-1006.
12. Teixeira, A.L., et al., *Higher circulating expression levels of miR-221 associated with poor overall survival in renal cell carcinoma patients.* Tumour Biol, 2014.35(5):p.4057-66
13. Lee, R.C., R.L. Feinbaum, and V. Ambros, *The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.* Cell, 1993. **75**(5): p. 843-854.
14. Pasquinelli, A.E., et al., *Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA.* Nature, 2000. **408**(6808): p. 86-89.

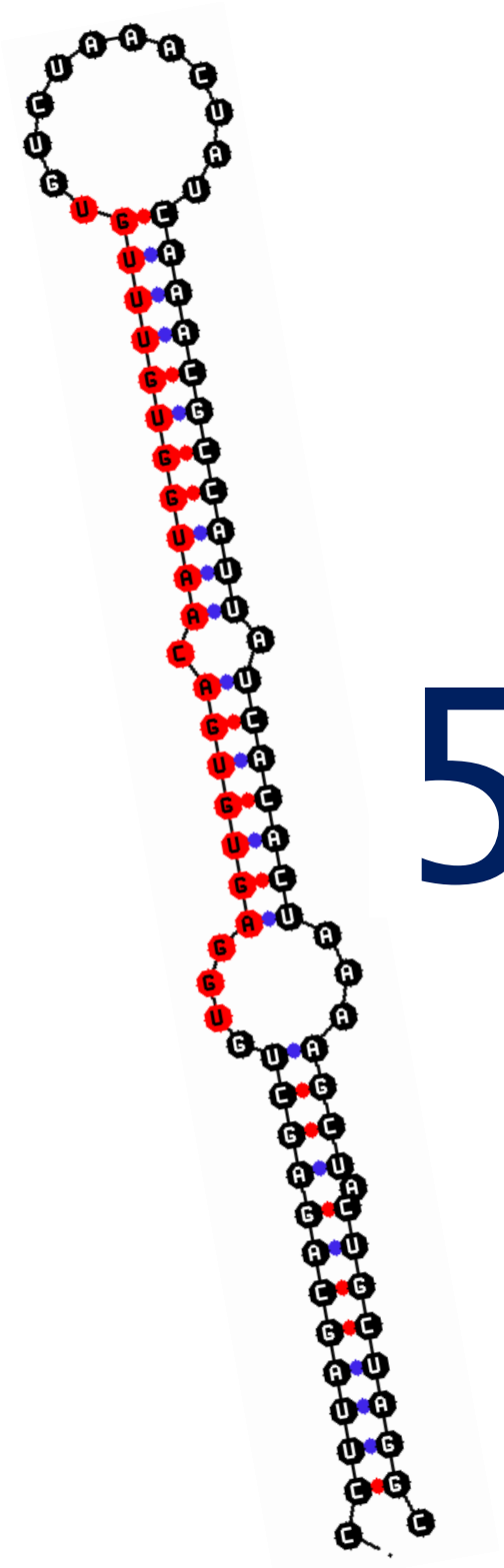
15. Vennemann, M. and A. Koppelkamm, *mRNA profiling in forensic genetics I: Possibilities and limitations.* Forensic Sci Int, 2010. **203**(1-3): p. 71-5.
16. Hanson, E.K., H. Lubenow, and J. Ballantyne, *Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs.* Anal Biochem, 2009. **387**(2): p. 303-14.
17. Zubakov, D., et al., *MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation.* Int J Legal Med, 2010. **124**(3): p. 217-26.
18. Weber, J.A., et al., *The microRNA spectrum in 12 body fluids.* Clin Chem, 2010. **56**(11): p. 1733-41.
19. Courts, C. and B. Madea, *Specific micro-RNA signatures for the detection of saliva and blood in forensic body-fluid identification.* J Forensic Sci, 2011. **56**(6): p. 1464-70.
20. Wang, Z., et al., *A model for data analysis of microRNA expression in forensic body fluid identification.* Forensic Sci Int Genet, 2012. **6**(3): p. 419-23.
21. Omelia, E.J., M.L. Uchimoto, and G. Williams, *Quantitative PCR analysis of blood- and saliva-specific microRNA markers following solid-phase DNA extraction.* Analytical Biochemistry, 2013. **435**(2): p. 120-122.
22. Courts, C. and B. Madea, *Micro-RNA - A potential for forensic science?* Forensic Sci Int, 2010. **203**(1-3): p. 106-11.
23. Winter, J. and S. Diederichs, *Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization.* RNA Biol, 2011. **8**(6): p. 1149-57.
24. An, J.-H., et al., *Body fluid identification in forensics.* BMB reports, 2012. **45**(10): p. 545-553.
25. Etheridge, A., et al., *Extracellular microRNA: A new source of biomarkers.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2011. **717**(1-2): p. 85-90.
26. Tilstone, W.J., K.A. Savage, and L.A. Clark, *Forensic Science: An Encyclopedia of History, Methods, and Techniques*. 2006: ABC-CLIO.
27. Moeller, K.E., K.C. Lee, and J.C. Kissack, *Urine Drug Screening: Practical Guide for Clinicians.* Mayo Clinic Proceedings, 2008. **83**(1): p. 66-76.
28. Ibberson, D., et al., *RNA degradation compromises the reliability of microRNA expression profiling.* BMC Biotechnol, 2009. **9**: p. 102.
29. Accerbi, M., et al., *Methods for isolation of total RNA to recover miRNAs and other small RNAs from diverse species.* Methods Mol Biol, 2010. **592**: p. 31-50.
30. Patnaik, S.K., R. Mallick, and S. Yendamuri, *Detection of microRNAs in dried serum blots.* Anal Biochem, 2010. **407**(1): p. 147-9.
31. Grasedieck, S., et al., *Impact of serum storage conditions on microRNA stability.* Leukemia, 2012. **26**(11): p. 2414-6.
32. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10513-8.
33. Shen, J., et al., *Plasma microRNAs as potential biomarkers for non-small-cell lung cancer.* Lab Invest, 2011. **91**(4): p. 579-87.
34. Gidlof, O., et al., *Cardiospecific microRNA plasma levels correlate with troponin and cardiac function in patients with ST elevation myocardial infarction, are selectively dependent on renal elimination, and can be detected in urine samples.* Cardiology, 2011. **118**(4): p. 217-26.
35. Varallyay, E., J. Burgyan, and Z. Havelda, *MicroRNA detection by northern blotting using locked nucleic acid probes.* Nat Protoc, 2008. **3**(2): p. 190-6.
36. Varallyay, E., J. Burgyan, and Z. Havelda, *Detection of microRNAs by Northern blot analyses using LNA probes.* Methods, 2007. **43**(2): p. 140-5.
37. Varallyay, E. and Z. Havelda, *Detection of microRNAs in plants by in situ hybridisation.* Methods Mol Biol, 2011. **732**: p. 9-23.
38. Liu, C.G., et al., *MicroRNA expression profiling using microarrays.* Nat Protoc, 2008. **3**(4): p. 563-78.
39. Berezikov, E., E. Cuppen, and R.H.A. Plasterk, *Approaches to microRNA discovery.* Nat Genet, 2006.38 Suppl: p.S2-7.

40. Wang, Z., M. Gerstein, and M. Snyder, *RNA-Seq: a revolutionary tool for transcriptomics.* Nat Rev Genet, 2009. **10**(1): p. 57-63.
41. Benes, V. and M. Castoldi, *Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available.* Methods, 2010. **50**(4): p. 244-9.
42. Rasmussen, R., *Quantification on the LightCycler*, in *Rapid Cycle Real-Time PCR*, S. Meuer, C. Wittwer, and K.-I. Nakagawara, Editors. 2001, Springer Berlin Heidelberg. p. 21-34.
43. Meyer, S.U., M.W. Pfaffl, and S.E. Ulbrich, *Normalization strategies for microRNA profiling experiments: a 'normal' way to a hidden layer of complexity?* Biotechnol Lett, 2010. **32**(12): p. 1777-88.
44. Duttagupta, R., et al., *Impact of cellular miRNAs on circulating miRNA biomarker signatures.* PLoS One, 2011. **6**(6): p. e20769.
45. Wang, K., et al., *Comparing the MicroRNA spectrum between serum and plasma.* PLoS One, 2012. **7**(7): p. e41561.
46. Wang, Y.T., et al., *Circulating microRNAs have a sex-specific association with metabolic syndrome.* J Biomed Sci, 2013. **20**: p. 72.
47. Luo, S.S., et al., *Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes.* Biol Reprod, 2009. **81**(4): p. 717-29.
48. Chim, S.S., et al., *Detection and characterization of placental microRNAs in maternal plasma.* Clin Chem, 2008. **54**(3): p. 482-90.
49. Miura, K., et al., *Identification of pregnancy-associated microRNAs in maternal plasma.* Clin Chem, 2010. **56**(11): p. 1767-71.
50. Gilad, S., et al., *Serum microRNAs are promising novel biomarkers.* PLoS One, 2008. **3**(9): p. e3148.
51. Morales Prieto, D.M. and U.R. Markert, *MicroRNAs in pregnancy.* J Reprod Immunol, 2011. **88**(2): p. 106-11.
52. Chiu, R.W. and Y.M. Lo, *Pregnancy-associated microRNAs in maternal plasma: a channel for fetal-maternal communication?* Clin Chem, 2010. **56**(11): p. 1656-7.
53. Kotlabova, K., J. Doucha, and I. Hromadnikova, *Placental-specific microRNA in maternal circulation--identification of appropriate pregnancy-associated microRNAs with diagnostic potential.* J Reprod Immunol, 2011. **89**(2): p. 185-91.
54. Mayor-Lynn, K., et al., *Expression profile of microRNAs and mRNAs in human placentas from pregnancies complicated by preeclampsia and preterm labor.* Reprod Sci, 2011. **18**(1): p. 46-56.
55. Mouillet, J.F., et al., *MiR-205 silences MED1 in hypoxic primary human trophoblasts.* FASEB J, 2010. **24**(6): p. 2030-9.
56. Morales-Prieto, D.M., et al., *MicroRNA expression profiles of trophoblastic cells.* Placenta, 2012. **33**(9): p. 725-34.
57. Ibanez-Ventoso, C., et al., *Modulated microRNA expression during adult lifespan in Caenorhabditis elegans.* Aging Cell, 2006. **5**(3): p. 235-46.
58. Smith-Vikos, T. and F.J. Slack, *MicroRNAs and their roles in aging.* J Cell Sci, 2012. **125**(Pt 1): p. 7-17.
59. Noren Hooten, N., et al., *microRNA expression patterns reveal differential expression of target genes with age.* PLoS One, 2010. **5**(5): p. e10724.
60. Noren Hooten, N., et al., *Age-related changes in microRNA levels in serum.* Aging (Albany NY), 2013. **5**(10): p. 725-40.
61. Boehm, M. and F. Slack, *A developmental timing microRNA and its target regulate life span in C. elegans.* Science, 2005. **310**(5756): p. 1954-7.
62. Li, N., et al., *Increased expression of miR-34a and miR-93 in rat liver during aging, and their impact on the expression of Mgst1 and Sirt1.* Mech Ageing Dev, 2011. **132**(3): p. 75-85.
63. Maes, O.C., et al., *Murine microRNAs implicated in liver functions and aging process.* Mech Ageing Dev, 2008. **129**(9): p. 534-41.
64. Li, N., et al., *Up-regulation of key microRNAs, and inverse down-regulation of their predicted oxidative phosphorylation target genes, during aging in mouse brain.* Neurobiol Aging, 2011. **32**(5): p. 944-55.

65. Hamrick, M.W., et al., *The adipokine leptin increases skeletal muscle mass and significantly alters skeletal muscle miRNA expression profile in aged mice.* Biochem Biophys Res Commun, 2010. **400**(3): p. 379-83.
66. Drummond, M.J., et al., *Aging and microRNA expression in human skeletal muscle: a microarray and bioinformatics analysis.* Physiol Genomics, 2011. **43**(10): p. 595-603.
67. Somel, M., et al., *MicroRNA, mRNA, and protein expression link development and aging in human and macaque brain.* Genome Res, 2010. **20**(9): p. 1207-18.
68. Moreau, M.P., et al., *Chronological changes in microRNA expression in the developing human brain.* PLoS One, 2013. **8**(4): p. e60480.
69. ElSharawy, A., et al., *Genome-wide miRNA signatures of human longevity.* Aging Cell, 2012. **11**(4): p. 607-16.
70. Hackl, M., et al., *miR-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging.* Aging Cell, 2010. **9**(2): p. 291-6.
71. Lee, H., et al., *Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in Arabidopsis.* Nucleic acids research, 2010. **38**(9): p. 3081-3093.
72. Sunkar, R. and J.K. Zhu, *Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis.* Plant Cell, 2004. **16**(8): p. 2001-19.
73. Liu, H.H., et al., *Microarray-based analysis of stress-regulated microRNAs in Arabidopsis thaliana.* RNA, 2008. **14**(5): p. 836-43.
74. de Lima, J.C., G. Loss-Morais, and R. Margis, *MicroRNAs play critical roles during plant development and in response to abiotic stresses.* Genet Mol Biol, 2012. **35**(4 (suppl)): p. 1069-77.
75. An, F.M., S.R. Hsiao, and M.T. Chan, *Sequencing-based approaches reveal low ambient temperature-responsive and tissue-specific microRNAs in phalaenopsis orchid.* PLoS One, 2011. **6**(5): p. e18937.
76. May, P., et al., *The effects of carbon dioxide and temperature on microRNA expression in Arabidopsis development.* Nat Commun, 2013. **4**: p. 2145.
77. Biggar, K.K., A. Dubuc, and K. Storey, *MicroRNA regulation below zero: differential expression of miRNA-21 and miRNA-16 during freezing in wood frogs.* Cryobiology, 2009. **59**(3): p. 317-21.
78. Morin, P., Jr., A. Dubuc, and K.B. Storey, *Differential expression of microRNA species in organs of hibernating ground squirrels: a role in translational suppression during torpor.* Biochim Biophys Acta, 2008. **1779**(10): p. 628-33.
79. Biggar, K.K., et al., *MicroRNA regulation in extreme environments: differential expression of microRNAs in the intertidal snail Littorina littorea during extended periods of freezing and anoxia.* Genomics Proteomics Bioinformatics, 2012. **10**(5): p. 302-9.
80. Benson, E.A. and T.C. Skaar, *Incubation of whole blood at room temperature does not alter the plasma concentrations of microRNA-16 and -223.* Drug Metab Dispos, 2013. **41**(10): p. 1778-81.
81. Jia, X., et al., *UV-B-responsive microRNAs in Populus tremula.* J Plant Physiol, 2009. **166**(18): p. 2046-57.
82. Wang, B., et al., *Identification of UV-B-induced microRNAs in wheat.* Genet Mol Res, 2013. **12**(4): p. 4213-21.
83. Templin, T., et al., *Whole mouse blood microRNA as biomarkers for exposure to gamma-rays and (56)Fe ion.* Int J Radiat Biol, 2011. **87**(7): p. 653-62.
84. Dressman, H.K., et al., *Gene expression signatures that predict radiation exposure in mice and humans.* PLoS Med, 2007. **4**(4): p. e106.
85. Kraemer, A., et al., *UVA and UVB irradiation differentially regulate microRNA expression in human primary keratinocytes.* PLoS One, 2013. **8**(12): p. e83392.
86. Templin, T., et al., *Radiation-induced micro-RNA expression changes in peripheral blood cells of radiotherapy patients.* Int J Radiat Oncol Biol Phys, 2011. **80**(2): p. 549-57.
87. Cui, W., et al., *Plasma miRNA as biomarkers for assessment of total-body radiation exposure dosimetry.* PLoS One, 2011. **6**(8): p. e22988.
88. Jacob, N.K., et al., *Identification of sensitive serum microRNA biomarkers for radiation biodosimetry.* PLoS One, 2013. **8**(2): p. e57603.

89. Zhao, B., et al., *Identification of drought-induced microRNAs in rice.* Biochem Biophys Res Commun, 2007. **354**(2): p. 585-90.
90. Sunkar, R., *MicroRNAs with macro-effects on plant stress responses.* Semin Cell Dev Biol, 2010. **21**(8): p. 805-11.
91. Ferreira, T.H., et al., *microRNAs associated with drought response in the bioenergy crop sugarcane (Saccharum spp.).* PLoS One, 2012. **7**(10): p. e46703.
92. Kantar, M., S.J. Lucas, and H. Budak, *miRNA expression patterns of Triticum dicoccoides in response to shock drought stress.* Planta, 2011. **233**(3): p. 471-84.
93. Wu, C.W., K.K. Biggar, and K.B. Storey, *Dehydration mediated microRNA response in the African clawed frog Xenopus laevis.* Gene, 2013. **529**(2): p. 269-75.
94. Welberg, L., *Neurodegenerative disorders: reconnect with microRNA.* Nat Rev Neurosci, 2010. **11**(2): p. 74.
95. Nelson, P.T., W.X. Wang, and B.W. Rajeev, *MicroRNAs (miRNAs) in neurodegenerative diseases.* Brain Pathol, 2008. **18**(1): p. 130-8.
96. Abe, M. and N.M. Bonini, *MicroRNAs and neurodegeneration: role and impact.* Trends Cell Biol, 2013. **23**(1): p. 30-6.
97. Hebert, S.S. and B. De Strooper, *Molecular biology. miRNAs in neurodegeneration.* Science, 2007. **317**(5842): p. 1179-80.
98. Lehmann, S.M., et al., *An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration.* Nat Neurosci, 2012. **15**(6): p. 827-35.
99. van Rooij, E., *The Art of MicroRNA Research.* Circulation Research, 2011. **108**(2): p. 219-234.
100. Kaeuferle, T., et al., *MicroRNA Methodology: Advances in miRNA Technologies.* Methods Mol Biol, 2014. **1169**: p. 121-30.
101. Inose, H., et al., *A microRNA regulatory mechanism of osteoblast differentiation.* Proceedings of the National Academy of Sciences, 2009. **106**(49): p. 20794-20799.
102. Huang, J., et al., *Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes.* Nat Med, 2007. **13**(10): p. 1241-7.
103. Xiao, B., et al., *Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response.* J Infect Dis, 2009. **200**(6): p. 916-25.
104. Marquez, R.T., et al., *Correlation between microRNA expression levels and clinical parameters associated with chronic hepatitis C viral infection in humans.* Lab Invest, 2010. **90**(12): p. 1727-36.
105. Kulkarni, S., et al., *Differential microRNA regulation of HLA-C expression and its association with HIV control.* Nature, 2011. **472**(7344): p. 495-8.
106. Esquela-Kerscher, A. and F.J. Slack, *Oncomirs - microRNAs with a role in cancer.* Nat Rev Cancer, 2006. **6**(4): p. 259-69.
107. Liu, X.G., et al., *High expression of serum miR-21 and tumor miR-200c associated with poor prognosis in patients with lung cancer.* Med Oncol, 2012. **29**(2): p. 618-26.
108. Fayyad-Kazan, H., et al., *Circulating miR-150 and miR-342 in plasma are novel potential biomarkers for acute myeloid leukemia.* J Transl Med, 2013. **11**: p. 31.
109. Hanke, M., et al., *A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer.* Urol Oncol, 2010. **28**(6): p. 655-61.
110. Yamada, Y., et al., *MiR-96 and miR-183 detection in urine serve as potential tumor markers of urothelial carcinoma: correlation with stage and grade, and comparison with urinary cytology.* Cancer Sci, 2011. **102**(3): p. 522-9.
111. Zeng, R.C., et al., *Down-regulation of miRNA-30a in human plasma is a novel marker for breast cancer.* Med Oncol, 2013. **30**(1): p. 477.
112. Ng, E.K., et al., *Circulating microRNAs as specific biomarkers for breast cancer detection.* PLoS One, 2013. **8**(1): p. e53141.
113. Madhavan, D., et al., *Circulating miRNAs as surrogate markers for circulating tumor cells and prognostic markers in metastatic breast cancer.* Clin Cancer Res, 2012. **18**(21): p. 5972-82.
114. Si, H., et al., *Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer.* J Cancer Res Clin Oncol, 2013. **139**(2): p. 223-9.

115. Wang, F., et al., *Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor.* Gynecol Oncol, 2010. **119**(3): p. 586-93.
116. Chen, W., et al., *The level of circulating miRNA-10b and miRNA-373 in detecting lymph node metastasis of breast cancer: potential biomarkers.* Tumour Biol, 2013. **34**(1): p. 455-62.
117. Cuk, K., et al., *Circulating microRNAs in plasma as early detection markers for breast cancer.* Int J Cancer, 2013. **132**(7): p. 1602-12.
118. Roth, C., et al., *Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer.* Breast Cancer Res, 2010. **12**(6): p. R90.
119. Mar-Aguilar, F., et al., *Serum circulating microRNA profiling for identification of potential breast cancer biomarkers.* Dis Markers, 2013. **34**(3): p. 163-9.
120. van Schooneveld, E., et al., *Expression profiling of cancerous and normal breast tissues identifies microRNAs that are differentially expressed in serum from patients with (metastatic) breast cancer and healthy volunteers.* Breast Cancer Res, 2012. **14**(1): p. R34.
121. Cheng, H., et al., *Circulating plasma MiR-141 is a novel biomarker for metastatic colon cancer and predicts poor prognosis.* PLoS One, 2011. **6**(3): p. e17745.
122. Zhang, C., et al., *Expression profile of microRNAs in serum: a fingerprint for esophageal squamous cell carcinoma.* Clin Chem, 2010. **56**(12): p. 1871-9.
123. Tsujiura, M., et al., *Circulating microRNAs in plasma of patients with gastric cancers.* Br J Cancer, 2010. **102**(7): p. 1174-9.
124. Cai, H., et al., *Plasma microRNAs serve as novel potential biomarkers for early detection of gastric cancer.* Med Oncol, 2013. **30**(1): p. 452.
125. Liu, R., et al., *A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis.* Eur J Cancer, 2011. **47**(5): p. 784-91.
126. Silva, J., et al., *Vesicle-related microRNAs in plasma of nonsmall cell lung cancer patients and correlation with survival.* Eur Respir J, 2011. **37**(3): p. 617-23.
127. Shen, J., et al., *Diagnosis of lung cancer in individuals with solitary pulmonary nodules by plasma microRNA biomarkers.* BMC Cancer, 2011. **11**: p. 374.
128. Boeri, M., et al., *MicroRNA signatures in tissues and plasma predict development and prognosis of computed tomography detected lung cancer.* Proc Natl Acad Sci U S A, 2011. **108**(9): p. 3713-8.
129. Zheng, D., et al., *Plasma microRNAs as novel biomarkers for early detection of lung cancer.* Int J Clin Exp Pathol, 2011. **4**(6): p. 575-86.
130. Heegaard, N.H., et al., *Circulating micro-RNA expression profiles in early stage nonsmall cell lung cancer.* Int J Cancer, 2012. **130**(6): p. 1378-86.
131. Roth, C., et al., *Screening for circulating nucleic acids and caspase activity in the peripheral blood as potential diagnostic tools in lung cancer.* Mol Oncol, 2011. **5**(3): p. 281-91.
132. Chen, Z.H., et al., *A panel of five circulating microRNAs as potential biomarkers for prostate cancer.* Prostate, 2012. **72**(13): p. 1443-52.
133. Mahn, R., et al., *Circulating microRNAs (miRNA) in serum of patients with prostate cancer.* Urology, 2011. **77**(5): p. 1265 e9-16.
134. Keller, A., et al., *Stable serum miRNA profiles as potential tool for non-invasive lung cancer diagnosis.* RNA Biology, 2011. **8**(3): p. 506-516.
135. Abd-El-Fattah, A., et al., *Differential MicroRNAs Expression in Serum of Patients with Lung Cancer, Pulmonary Tuberculosis, and Pneumonia.* Cell Biochemistry and Biophysics, 2013. **67**(3): p. 875-884.
136. Le, H.B., et al., *Evaluation of dynamic change of serum miR-21 and miR-24 in pre- and post-operative lung carcinoma patients.* Med Oncol, 2012. **29**(5): p. 3190-7.



5 Chapter 2

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Submitted

Forensic Science International: Genetics

Circulating miRNA signatures for blood and urine identification.

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Abstract

In forensics, the identification of blood, semen or vaginal secretions can represent an important support in a criminal investigation. Those biological samples can be used as a source of DNA but also can hold, only by their presence, the most probative value. Throughout many years, many methodologies were used to identify them but all presented serious drawbacks. Lately, mRNA surged as a potential tool for body fluid identification but their sensibility to both biotic and abiotic factors were a serious disadvantage, even more pronounced in forensic samples. Since 2009, miRNA profiling surged as a possible emergent tool as a confirmatory test in forensics due to their tissue specific pattern of expression. Unlike mRNAs they are much more stable due to their properties whose makes them less susceptible to degradation processes.

In this report, we studied the relative expression patterns of miR-127, miR-221, miR-222 and RNU-48 in 50 individuals samples (urine and blood) in order to define whether or not those could be used as biomarkers for urine and blood identification.

In addition to evaluate whether or not our miRNAs could be considered as biomarkers, we came across with two others conclusions: the impact of miRNA purity in miRNAs relative quantification and the adequate selection of the normalization gene for blood and urine identification.

Key words: miRNA profiling, Forensic, Serology, body fluids, biological biomarkers

1- Introduction

Human body fluids are important components to rely on a criminal investigation [1-4]. As a matter of fact, a complainant's body fluids present on items belonging to a suspect – or vice versa – holds the most probative value [5]. For example, in a case of an alleged sexual assault in a child, the DNA that was recovered from the child's bedding and underwear was coincident with his father DNA profile. Can we consider his father responsible for the sexual assault? In a situation like this, it is not enough to recover a DNA profile but it is also imperative to acknowledge its source. If no serological test were done, in court, the presence of DNA could be explained as a result of the presence of epithelial cells in the child clothing which is totally common when it comes from a sibling. On the other hand, if serological tests linked the DNA profile to semen it would be way more difficult to explain its presence there.

Beyond the probative value that body fluids may have in a crime scene, it is also important to acknowledge them in order to use optimized protocols to conduct a reliable DNA profiling [6, 7]. For example, DNA extraction processes are different to blood and urine. If we conducted the protocol of blood extraction in urine samples, it may result in a reduced quality of the extracted DNA e enable any conclusive DNA profile [6, 7]. For those reasons, reliable detection and identification of body fluids are considered crucial step in criminal investigation.

Apparently, it seems easy to identify body fluids such as blood (colour), urine (smell) or even sperm (texture) due to their specific characteristics. However, when dried, washed or mixed with other components, their identification may not be that easy [1, 5, 8]. It is important to highlight that in court, there is no such thing as "It seems to be sperm because it looked like it and have the same particular texture", it is needed an undeniable proof that it is sperm. Serological tests are used in forensic biology to allow the detection and identification of body fluids in both native form or as a residue left at a crime scene [4]. Serological tests are divided in two major fields: presumptive and confirmatory tests [9-15]. Presumptive tests rely on methodologies that are sensitive and performed quickly, yet they are not specific to the body fluid. Those tests can only indicate if the fluids might be present and do not unequivocally states its presence [5, 16]. On the other hand, confirmatory tests are indeed

specific to the body fluid we seek to identify. As presumptive tests, confirmatory testing is sensitive however, it takes a lot more time to perform [5, 16]. Idealistically, we should have a battery of confirmatory test for all important body fluids in order to reliably detect and identify them. Unfortunately, there is a large cluster of presumptive tests and far less number of confirmatory ones. Moreover, till date no confirmatory test is able to reliably differentiate blood from menstrual blood which is an unquestionably important body fluid in sexual crimes cases.

Over the last years, messenger RNA (mRNA) profiling became a target for body fluid identification due to its tissue specific patterns [17-19]. Still, mRNA susceptibility to degradation by physical or chemical factors was an unquestionable drawback [3]. In order to sidetrack this problem, microRNAs (miRNAs) surge with a real potential as a confirmatory test [20]. MiRNAs are small non-coding RNAs with more or less than 22 nucleotides of length that, combined with the RNA-induced silencing complex, seems to regulate a major part of human gene expression [21-23]. Their regulatory functions have been recognized to be responsible for several biological processes such as apoptosis, differentiation and proliferation but also have a significant role in pathological conditions [24-29]. Most importantly, their tight relationship with Argonaute proteins makes them much less susceptive to both biotic and abiotic factors [30]. In 2009, Hanson and colleagues were the first to introduce miRNA profiling for body fluid identification in a forensic purpose and soon enough others followed [31-35]. Those studies pointed out a large collection of miRNAs with potential as biomarker, however very few were confirmed by more than one group which revealed the lack of reproducibility of results [36]. Besides, when some tried to replicate the results of others - they failed, highlighting the lack of a standard workflow of methodology that would be essential for miRNA profiling reliability [32].

In this report, we choose to test the identification and quantification of four miRNAs in both blood and urine samples obtained from 50 healthy individuals and study their behaviour within those body fluids, using a real-time PCR methodology.

2- Material and methods

Study population

We conducted a miRNA expression profiling study in 50 healthy adult individuals (34% males and 66% females) from North of Portugal. Our group of study was composed by Caucasian individuals with a mean age of 42.7 years, with no major pathological condition in order to erase a variable that could alter miRNAs expression profile. Peripheral venous blood (6ml) and urine samples were collected from each subject following the obtainment of a written informed consent from all subjects according the Helsinki Declaration principles.

miRNA extraction/purification

After collected the biologic samples, they were processed according the following experimental conditions. For urine samples, the pellet resulting from a 20 minute centrifugation at 3000 rpm was diluted with 1X PBS (Phosphate buffered saline), and them centrifuged during 15 minutes at 3000 rpm, at room temperature. The pellet was then stabilized with TriPure® Isolation Reagent (Roche Applied Science) and preserved at -80ºC. For blood, the samples were centrifuged during 5 minutes at 2500 rpm at room temperature. We transferred fresh blood in a 50 mL falcon and add 1xAKE (ammonium chloride, potassium hydrogen phosphate and EDTA). We froze the samples during 20 minutes at -20ºC and afterwards centrifuged them during 10 minutes at 2500 rpm. To the pellet was added 1xAKE and the mixture centrifuged during 10 minutes at 2500rpm. The pellet was suspended with 30 ml of 1xPBS which posteriorly, was centrifuge during 10 minutes at 2500rpm and the resulting pellet diluted in 1ml of TriPure® Isolation Reagent (Roche Applied Science). In urine samples, 200µl of processed samples was added to 8µl of miRNA buffer and 80µl de chloroform. The solution incubated 10 minutes at room temperature and centrifuged at 1200g during 15 minutes. To the resulting upper-phase it was added 43,2 µl of absolute ethanol. To this point forward we handled the samples with GRS microRNA Kit (Grisp®) according to the manufacturer's instructions yet, we used half the volumes. The blood samples were handled for miRNAs extraction using the GRS microRNA Kit (Grisp®) according to the manufacturer's instructions.

cDNA synthesis and miRNA relative quantification

Subsequently, miRNAs previously extracted were used as a template for cDNA synthesis using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®). Afterward, *NanoDrop® ND-1000* was used to measure UV

absorbance spectra determining 260/280nm ratios in order to assess the quantity and quality of RNA.

To quantify miRNA expression, real-time PCR assays were performed with a StepOne™ System, reactions were carried out 1X TaqMan®Universal Master Mix II (Applied Biosystems®), with 1X probes to amplify the target miRNAs (Taqman MicroRNA Assays miR-127: 002229, miR-221: 002096, miR-222: 002097 and RNU-48: 002745 and cDNA sample.

The data analysis was carried out using the StepOne Software v2.2 (Applied Biosystems®) with the same baseline and threshold set for each plate, in order to generate threshold cycle (Ct) values for all the genes in each sample.

Statistical Analysis

Data analysis was performed by the computer software IBM®SPSS®Statistics (Version 22.0). In order to assess any statistical alterations in our normalized miRNAs expression essay we used $2^{-\Delta\Delta Ct}$ method along with Student's t test was used in order to evaluate any statistical differences in miRNA expression here explored.

3- Results and Discussion

3.1- Cycle threshold vs miRNA purity

Urine samples were processed and the resulting pellet was diluted in 1ml of TriPure® Isolation Reagent (Roche Applied Science®). Visually a wide range of pink colour was noticeable within our urine samples. Those with a deep pink were related with samples with a more substantial pellet unlike those with a less considerable pellet who presented themselves with a lighter colour. After miRNA extraction, we quantify miR-222 in urine samples and perceived that only few of them were detected. Interestingly, only the ones with a lighter colour were indeed detected. This tricky situation was associated with the ratio of absorbance (260 nm/280nm) which is used to assess the purity of RNA [37]. We observed that lighter colour was an indicator of a high ratio, on the other hand, those with higher optical density had a very low ratio, far from the ratio of ~2.0 which is generally accepted as “pure” for RNA [37]. In order to sidetrack this situation, we choose a sample that has an optimal 260/280nm ratio and diluted the other samples to equalize its optical density with TriPure® Isolation Reagent (Roche Applied Science®). Posteriorly, we choose 5 samples to test and observed a

considerable decrease of Ct in the samples processed with the optimized protocol (Fig.1). By definition the cycle threshold (Ct) is the number of cycle required for the fluorescent signal to overpass the threshold. Its levels decrease proportionally with the increase of the target in the sample [38].

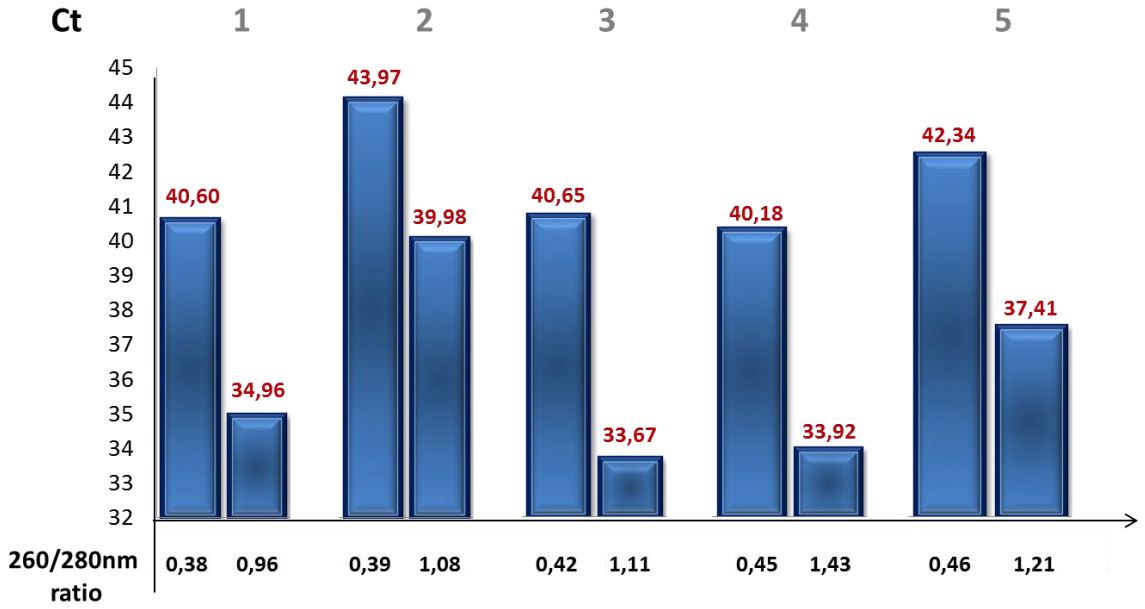

Figure 1 *- Cycle threshold vs RNA purity. This figure presents the Ct values of miR-222 taken from 5 samples processed with both normal and optimized protocol (first and second column respectively). It is showed that the considerable fall of Ct values correlates with an increase of 260/280nm ratio.*

The difference of Ct value is very significant once, at each cycle, the amount of DNA is duplicated, demonstrating that RNA purity is clearly a factor that challenges miRNA profiling. As showed, miRNA quantification can present itself with a low concentration or can go totally undetected when 260/280nm ratio is low however, when optimized, miRNA concentration increased significantly. As said previously, different reports indicated miRNAs as biomarkers for human body fluids identification though, when others tried to replicate them, they failed. Our results show that for the same sample, different degrees of purity are crucial for their positive detection. There is why, RNA purity needed to be optimal, otherwise it may lead to unreliable results, which could explain, the failed attempts done by some authors when trying to replicate others results.

3.2 – Endogenous control selection

In qRT-PCR, data normalization is imperatively required for relative quantification analysis [39-41]. The integration of an invariant endogenous -

control, also called as reference gene, has the main purpose to correct systematic technical and/or experimental errors [40, 42]. Thus, we choose to use RNU-48 as our reference gene for the data normalization. Widely used as an endogenous control, RNU-48 is expected to have a stable pattern among samples [43, 44]. However, according our results, within our assays we didn't observe this stable pattern between the samples types included in this study. As showed in figure 2, RNU-48 was the one with a major standard deviation, between blood and urine samples, when compared with the other 3 miRNAs analyzed, which make it inappropriate as an endogenous control for our study.

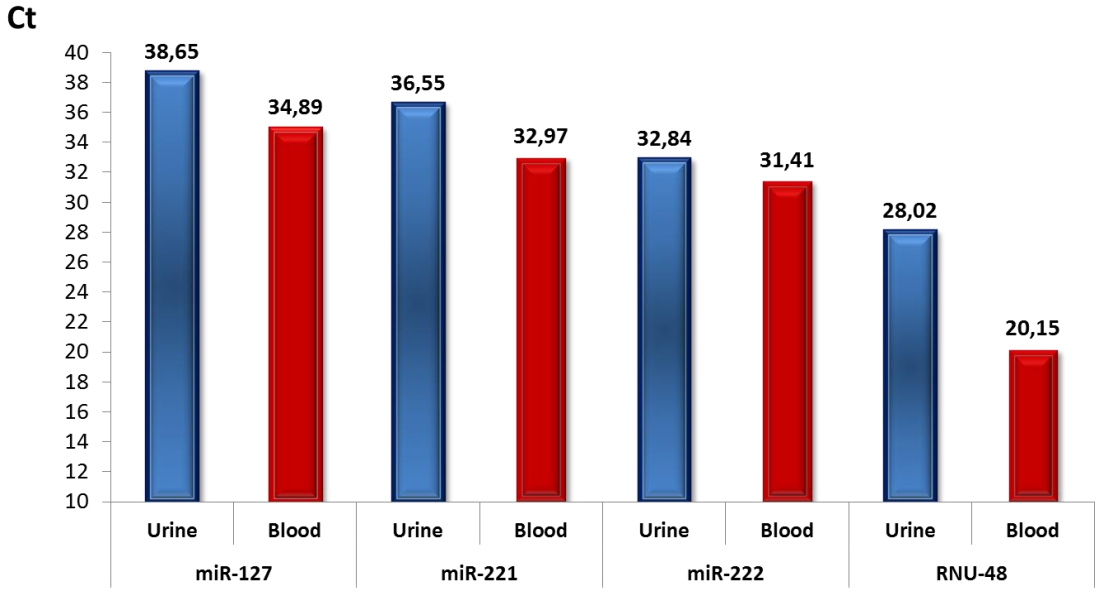

***Figure 2** – Average Ct of both urine and blood samples for all four miRNAs considered for this report. Interestingly, RNU-48 – our first choice as endogenous control - is the one presenting the most considerable standard deviation. On the other hand, miR-222 behaves more like a reference gene once it does not present a significant change of profile between urine and blood.*

Seemingly, we were not the only ones that concluded this variation, Sapre et al. also assumed that RNU-48 was inadequate as an endogenous control due to its systematic perturbation in its expression levels [45]. Ultimately, what we want to highlight is the important to define a correct normalization gene depending on the samples we choose to work with. Here, we do not discredit RNU-48 as a normalization gene however, when it comes to urine and blood profiling it is not reliable for data normalization.

Remarkably, the unexpected miR-222 profile remained barely unaffected and presented no significant differences between urine and blood. miR-222 behaviour within our samples was surprising once, it is being aimed for its deregulation by many other groups [46-48]. Here, it does not present any

variation within samples, any variation among both body fluids, it did even remained stable within different stages of age and do not alter with gender. According the literature, this particular behaviour is expected of endogenous controls, thus we decided to use miR-222 as our endogenous control in order to normalise our data in the present study [49].

3.3 – miRNAs as body fluids biomarkers

Attending the behaviour of miR-222, we used it as an endogenous control for our data normalisation in order to analyse differences in the expression levels of miR-127, miR-221 and RNU-48 among blood and urine samples.

As showed in figure 3, we can state that all miRNAs considered were detected at variable levels in blood and urine samples. We observed statistical significant higher expression levels of miR-127, miR-221 and RNU-48 in blood samples than in urine samples ($P<0.050$).

In table 1, we assessed the fold change in expression levels of the miRNAs included in the study and concluded that miR-221 has a 4,86 fold increase in blood when compared to urine while miR-221 has 5,39 fold increase.

Table 1 *– miRNA detection in both urine and blood samples and its corresponding fold change within the body fluids.*
**$\Delta\Delta Ct = (\Delta Ct_{blood} - \Delta Ct_{urine})$*

	Mean ΔCt urine	Mean ΔCt blood	ΔΔCt *	Fold Change	*P*-value
miR-127	38,65	34,89	-2,43	5,388934	0,000
miR-221	36,55	32,97	-2,28	4,856780	0,000
RNU-48	28,02	20,15	-7,14	141,043855	0,000

RNU-48 is the one with a major difference between urine and blood. This miRNA used numerous times as an endogenous control present a 141-fold-increase expression in blood when compared to urine, supporting our decision to not use it to normalize our data.

Currently, a minor number of miRNAs have been acknowledged as tissue specific - at least reliably. In the literature, miRNAs are considered tissue specific when they're found with high abundance in a specific tissue while it has low or non-existent expression in others. The definition itself is questionable, once there are no values that define what "high abundance" and "low-abundance" are. It is imperative to define a range of values to define those terms and by that, lead to reliable results. That differential profile patterns would allow body fluids identification and serve as a major confirmatory test. Considering our results, we can conclude that miR-127, miR-221 and RNU-48 are not suitable for neither

blood nor urine identification. Despite a significant difference of expression, they do not present the expected gap of expressional patterns to be considered as a good biomarker.

As we previously stated, the miRNAs considered as biomarkers for body fluid identification in other reports have been difficult to replicate. We admit that those difficulties are linked to several factors as environmental factors, different methodologies used, age, gender, pathologies among several others. We know that miRNAs expression levels do alter with both biotic and abiotic factors, there is why we try to minimize the impact of those within our samples excluding, as example, acute pathological conditions. Despite considering that miR-127, miR-221 and RNU-48 are unsuitable for urine and blood identification, we wanted to study their expressional behavior within samples with different stages of age and gender. Figure 4A displays an overview of their relative quantification within female and male samples. Considering blood samples, we did not observe any statistical significant difference in miRNAs expression (P>0,050). On the other hand, in urine, RNU-48 presented itself with a significant overexpression in females (P<0,050). Once again, this overexpression presented itself as another drawback to the use of RNU-48 as endogenous control.

When it comes to age, we divided our sampling in 3 age groups: 20-40, 41-60 and over 60 years old. As it is shown in figure 4B, the sample relative quantification demonstrated no statistical significant differences in their expression profile – neither in blood nor in urine (P>0,050).

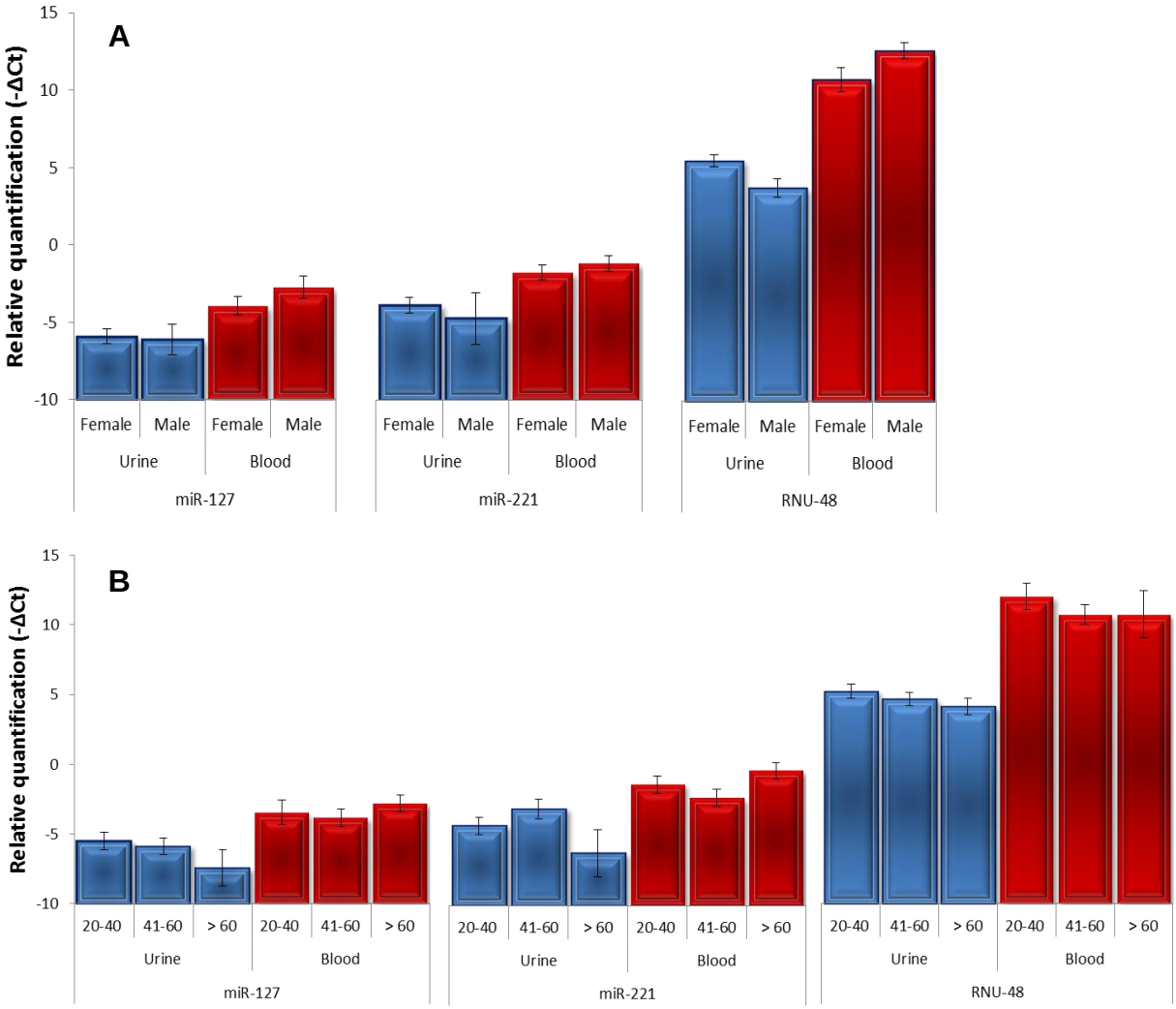

Figure 3 *– Relative quantification of miR-127, miR-221 and RNU-48 when conditioned by age and gender.*

4 – Conclusion and future perspectives

More than just a source of DNA, body fluids sole presence can have the most probative value. Hanson et al. introduced miRNA profiling as a reliable tool to identify body fluids such as blood, menstrual blood, semen, vaginal secretion and urine due to their tissue-specific pattern and stability when conditioned by degradation processes [18, 50].

Here we focused our attention in four miRNAs: miR-127, miR-221, miR-222 and RNU-48. Soon enough miRNAs purity struck our attention, when we observe that low value of 260/280nm ratio was associated with a poor degree of detection. When we upgraded our miRNA isolation protocol the consequence reflected in a higher success detection rate and in a considerable decrease of the samples Cycle threshold.

It would be irrefutably helpful to understand which threshold could affect miRNA profiling once, as it was shown, miRNA purity do affect considerably their relative quantification. It could even convey wrong outcomes once, even miRNAs with high concentration within body fluids can appear with low concentration or totally inexistent.

Our second result emphasised the importance of an adequate endogenous control selection attending the study objectives. Initially, according to the literature, we select the RNU-48 as our endogenous control but its behaviour within blood and urine make us reconsider our decision. RNU-48 is usually used as a reference gene due to its stable behaviour within samples however, our assay showed otherwise. Within the miRNAs tested, RNU-48 was the one with a more pronounced variability within the different samples, which is opposed of what would be expected of an endogenous control. Unexpectedly, miR-222 presented itself with the lowest standard deviation between blood and urine samples. Additionally, we studied its expression levels and compared them within age and gender and concluded that no significant alteration was noticeable. As stated earlier, endogenous controls are indispensable to validate qRT-PCR results however, till date, no endogenous control is universally acknowledged. This problem is reflected in our case, where one of the most used as an endogenous control proved to be unsuitable for urine and blood miRNA analysis. This subject is a very sensitive point in miRNA profiling. There is why it is imperative to focus our future line of work towards finding a reliable normalisation gene before anything else.

In this case, we could establish that all four miRNAs have different expressional patterns in urine and blood. However, to be considered as a biomarker, a major difference within body fluids would be expected. We conclude that the miRNAs analysed do not have the potential to be considered as a biomarker for body fluid identification.

Conflict of interest

None.

Acknowledgment

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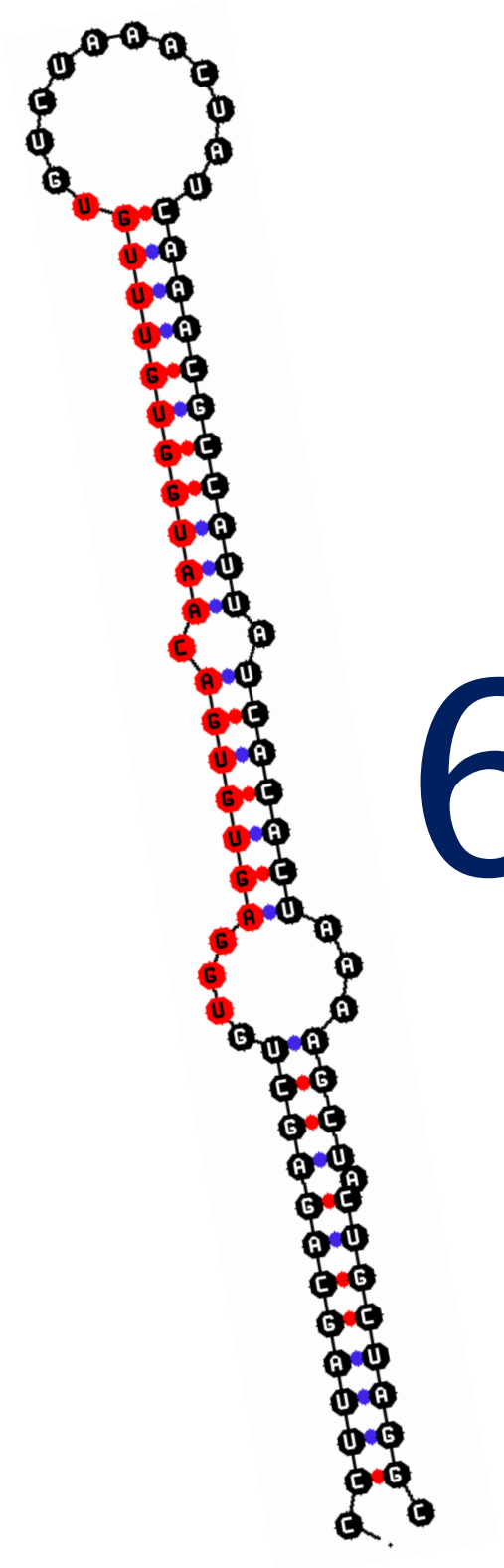
5 – References

1. Virkler, K. and I.K. Lednev, *Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene.* Forensic Science International, 2009. **188**(1–3): p. 1-17.
2. Fraser, J. and R. Williams, *Handbook of Forensic Science*. 2013: Taylor & Francis.
3. Park, J.L., et al., *Identification of body fluid-specific DNA methylation markers for use in forensic science.* Forensic Sci Int Genet, 2014. **13C**: p. 147-153.
4. An, J.H., et al., *Body fluid identification in forensics.* BMB Rep, 2012. **45**(10): p. 545-53.
5. Mozayani, A. and C. Noziglia, *The Forensic Laboratory Handbook Procedures and Practice*. 2010: Humana Press.
6. Butler, J.M., *Fundamentals of Forensic DNA Typing*. 2009: Elsevier Science.
7. McClintock, J.T., *Forensic Analysis of Biological Evidence: A Laboratory Guide for Serological and DNA Typing*. 2014: Taylor & Francis.
8. Butler, J.M., *Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers*. 2005: Elsevier Science.
9. Vennemann, M., et al., *Sensitivity and specificity of presumptive tests for blood, saliva and semen.* Forensic Sci Med Pathol, 2014. **10**(1): p. 69-75.
10. Patel, G. and A. Hopwood, *An evaluation of luminol formulations and their effect on DNA profiling*. Int J Legal Med, 2013. **127**(4): p. 723-9.
11. de Almeida, J.P., N. Glesse, and C. Bonorino, *Effect of presumptive tests reagents on human blood confirmatory tests and DNA analysis using real time polymerase chain reaction.* Forensic Sci Int, 2011. **206**(1-3): p. 58-61.
12. Giampaoli, S., et al., *Forensic interlaboratory evaluation of the ForFLUID kit for vaginal fluids identification.* Journal of Forensic and Legal Medicine. **21**: p. 60-63.
13. Lewis, J., et al., *Improved detection of semen by use of direct acid phosphatase testing.* Sci Justice, 2013. **53**(4): p. 385-94.
14. Ong, S.Y., et al., *Forensic identification of urine using the DMAC test: a method validation study.* Sci Justice, 2012. **52**(2): p. 90-5.
15. Goodwin, W., A. Linacre, and S. Hadi, *An Introduction to Forensic Genetics*. 2011: Wiley.
16. Bell, S., B.A.J. Fisher, and R.C. Shaler, *Encyclopedia of Forensic Science*. 2008: Facts On File.
17. Xu, Y., et al., *Development of Highly Sensitive and Specific mRNA Multiplex System (XCYR1) for Forensic Human Body Fluids and Tissues Identification.* PLoS ONE, 2014. **9**(7): p. e100123.
18. Vennemann, M. and A. Koppelkamm, *mRNA profiling in forensic genetics I: Possibilities and limitations.* Forensic Sci Int, 2010. **203**(1-3): p. 71-5.
19. Bauer, M., *RNA in forensic science.* Forensic Sci Int Genet, 2007. **1**(1): p. 69-74.
20. Li, Y., et al., *A strategy for co-analysis of microRNAs and DNA.* Forensic Science International: Genetics, 2014. **12**(0): p. 24-29.
21. Dias, F., et al., *Renal cell carcinoma development and miRNAs: a possible link to the EGFR pathway.* Pharmacogenomics, 2013. **14**(14): p. 1793-1803.
22. Jovanovic, M. and M.O. Hengartner, *miRNAs and apoptosis: RNAs to die for.* Oncogene, 0000. **25**(46): p. 6176-6187.
23. Krol, J., I. Loedige, and W. Filipowicz, *The widespread regulation of microRNA biogenesis, function and decay.* Nat Rev Genet, 2010. **11**(9): p. 597-610.
24. Pauli, A., J.L. Rinn, and A.F. Schier, *Non-coding RNAs as regulators of embryogenesis.* Nat Rev Genet, 2011. **12**(2): p. 136-149.
25. Esteller, M., *Non-coding RNAs in human disease.* Nat Rev Genet, 2011. **12**(12): p. 861-874.

26. Bartel, D.P., *MicroRNAs: Genomics, Biogenesis, Mechanism, and Function.* Cell, 2004. **116**(2): p. 281-297.
27. Teixeira, A.L., M. Gomes, and R. Medeiros, *EGFR signaling pathway and related-miRNAs in age-related diseases: the example of miR-221 and miR-222.* Frontiers in genetics, 2012. **3**.
28. Teixeira, A.L., et al., *Higher circulating expression levels of miR-221 associated with poor overall survival in renal cell carcinoma patients.* Tumor Biology, 2014. **35**(5): p. 4057-4066.
29. Teixeira, A., et al., *891 Circulating MicroRNA-222 in Plasma–a Potential Biomarker for Renal Cell Carcinoma.* European Journal of Cancer, 2012. **48**: p. S216.
30. Courts, C. and B. Madea, *Micro-RNA – A potential for forensic science?* Forensic Science International, 2010. **203**(1–3): p. 106-111.
31. Hanson, E.K., H. Lubenow, and J. Ballantyne, *Identification of forensically relevant body fluids using a panel of differentially expressed microRNAs.* Analytical Biochemistry, 2009. **387**(2): p. 303-314.
32. Zubakov, D., et al., *MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation.* International Journal of Legal Medicine, 2010. **124**(3): p. 217-226.
33. Wang, Z., et al., *A model for data analysis of microRNA expression in forensic body fluid identification.* Forensic Science International: Genetics, 2012. **6**(3): p. 419-423.
34. Weber, J.A., et al., *The MicroRNA Spectrum in 12 Body Fluids.* Clinical Chemistry, 2010. **56**(11): p. 1733-1741.
35. Courts, C. and B. Madea, *Specific Micro-RNA Signatures for the Detection of Saliva and Blood in Forensic Body-fluid Identification.* Journal of Forensic Sciences, 2011. **56**(6): p. 1464-1470.
36. Silva, S.S., et al., *Forensic miRNA: Potential biomarker for body fluids?* Forensic Science International: Genetics.
37. Rapley, R. and J.M. Walker, *Molecular Biomethods Handbook*. 2008: Humana Press.
38. Patrinos, G. and W. Ansorge, *Molecular Diagnostics*. 2005: Elsevier Science.
39. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT–PCR.* Nucleic Acids Research, 2001. **29**(9): p. e45.
40. Kozera, B. and M. Rapacz, *Reference genes in real-time PCR.* Journal of Applied Genetics, 2013. **54**(4): p. 391-406.
41. Guénin, S., et al., *Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references.* Journal of Experimental Botany, 2009. **60**(2): p. 487-493.
42. Meyer, S., M. Pfaffl, and S. Ulbrich, *Normalization strategies for microRNA profiling experiments: a 'normal' way to a hidden layer of complexity?* Biotechnology Letters, 2010. **32**(12): p. 1777-1788.
43. Linda Wong, K.L., Iain Russell, and Caifu Chen *Endogenous Controls for Real-Time Quantitation of miRNA Using TaqMan® MicroRNA Assays. .* 2010.
44. Gupta, R.C., *Biomarkers in Toxicology*. 2014: Elsevier Science.
45. Sapre N, H.M., Macintyre G, Lewis H, Kowalczyk A, et al., *Curated MicroRNAs in Urine and Blood Fail to Validate as Predictive Biomarkers for High-Risk Prostate Cancer.* PLOS ONE, 2014. **9**(4).
46. Zhang, D.Q., et al., *Increased expression of miR-222 is associated with poor prognosis in bladder cancer.* World J Surg Oncol, 2014. **12**(1): p. 241.
47. Mao, K.P., et al., *MicroRNA-222 expression and its prognostic potential in non-small cell lung cancer.* ScientificWorldJournal, 2014. **2014**: p. 908326.
48. Yang, Y.F., et al., *MiR-222 overexpression promotes proliferation of human hepatocellular carcinoma HepG2 cells by downregulating p27.* Int J Clin Exp Med, 2014. **7**(4): p. 893-902.
49. Bustin, S.A., *The PCR Revolution: Basic Technologies and Applications*. 2010: Cambridge University Press.
50. Courts, C. and B. Madea, *Micro-RNA - A potential for forensic science?* Forensic Sci Int, 2010. **203**(1-3): p. 106-11.



6 General Conclusion and Future Perspectives

6 - General conclusion and future perspectives

"Ah, not in knowledge is happiness, but in the acquisition of knowledge! In forever knowing, we are forever blessed."

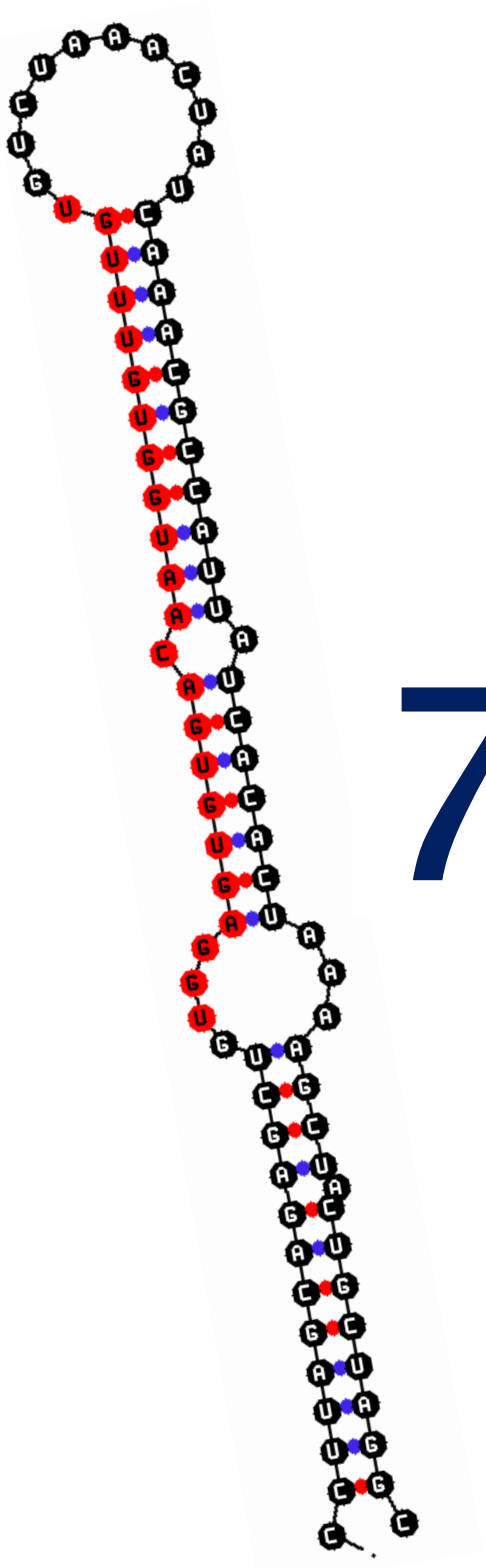
Edgar A. Poe

The importance of body fluids in forensics is undeniable, however the methodologies used for their detection and identification are not always as reliable that would be expected. Recently, miRNA profiling was introduced as a possible tool for their identification but very little has been done yet. Very few scientific reports have been published and their respective results not always reproducible.

We studied an idea that took form in 2009 and scientifically speaking, five years is such a short time – definitely too short to bring up a reliable methodology.

We proceed to a miRNA profiling study in urine and blood samples in a much larger sampling than used previously by other authors. We reached interesting conclusions namely the importance of factors that indirectly influence miRNA profiling within body fluids. Those results back up our previous statements that we published in the review article. Throughout our review, we were able to highlight potential variables, both biotic and abiotic, that could undermine miRNA full potential as a biomarker for human body-fluids identification. Moreover, we were able to highlight the impact of miRNA purity on their expressional profiling but also the impact of an adequate selection of the normalization gene for blood and urine identification.

Our conclusions lead to an imperative future line of work. It would be crucial to do a major screening study of the expressional levels of others miRNAs within body fluids, but also acknowledge the implications of both biotic and abiotic variables in miRNA profiling. Those studies would be essential to highlight their still latent potential.



7 References

7 - References

There is an eloquence in true enthusiasm

Edgar A. Poe

1. Bogusz, M.J., *Forensic Science*. 2000: Elsevier Science.
2. Kiely, T.F., *Forensic Evidence: Science and the Criminal Law, Second Edition*. 2005: Taylor & Francis.
3. Turvey, B.E., *Criminal Profiling: An Introduction to Behavioral Evidence Analysis*. 2011: Elsevier Science.
4. Horswell, J., *The Practice Of Crime Scene Investigation*. 2004: Taylor & Francis.
5. Science, C.D.N.A.T.F., et al., *DNA Technology in Forensic Science*. 1992: National Academies Press.
6. Dirkmaat, D.C., et al., *New perspectives in forensic anthropology.* Am J Phys Anthropol, 2008. **Suppl 47**: p. 33-52.
7. Amendt, J., R. Krettek, and R. Zehner, *Forensic entomology.* Naturwissenschaften, 2004. **91**(2): p. 51-65.
8. Gennard, D., *Forensic Entomology: An Introduction*. 2012: Wiley.
9. van Oorschot, R.A., K.N. Ballantyne, and R.J. Mitchell, *Forensic trace DNA: a review.* Investig Genet, 2010. **1**(1): p. 14.
10. Shankar, A.A. and R.C. Dandekar, *Forensic odontology as an aid for victim identification in mass disasters.* Dent Res J (Isfahan), 2012. **9**(1): p. 120-1.
11. Sweet, D., *Forensic dental identification.* Forensic science international, 2010. **201**(1): p. 3-4.
12. Sims, D.N., N.E. Langlois, and R.W. Byard, *An approach to peer review in forensic pathology.* J Forensic Leg Med, 2013. **20**(5): p. 402-3.
13. Smith, M.L., et al., *Modern instrumental methods in forensic toxicology.* J Anal Toxicol, 2007. **31**(5): p. 237-53, 8A-9A.
14. Ltd, D.K., *Forensic Science*. 2008: Dorling Kindersley Limited.
15. Mozayani, A. and C. Noziglia, *The Forensic Laboratory Handbook: Procedures and Practice*. 2007: Humana Press.
16. Fish, J.T., et al., *Crime Scene Investigation*. 2013: Elsevier Science.
17. Bertino, A., *Forensic Science: Fundamentals and Investigations 2012 Update*. 2011: Cengage Learning.
18. Segrave, K., *Lie Detectors: A Social History*. 2003: McFarland.
19. Woolf, G., *Et Tu, Brute?: The Murder of Caesar and Political Assassination*. 2006: Harvard University Press.
20. Erer, S., et al., *A Forensic Autopsy Case Belonging to the Nineteenth Century in Turkey.* Journal of the International Society for the History of Islamic Medicine (JISHIM), 2006. **5**: p. 40–45.
21. Lu, G.-D.a.N., Joseph, *A history of forensic medicine in China.* Medical History, 1988. **32**(04): p. 357-400.
22. Yeatts, T., *Forensics: Solving the Crime*. 2001: Oliver Press.
23. Kelly, D.C., *The Human Predator: A Historical Chronicle of Serial Murder and Forensic Investigation.* Journal of the American Academy of Psychiatry and the Law Online, 2008. **36**(3): p. 423-425.
24. James, S.H. and J.J. Nordby, *Forensic Science: An Introduction to Scientific and Investigative Techniques*. 2009: CRC Press/Taylor & Francis Group.
25. Brown, R. and J. Davenport, *Forensic Science: Advanced Investigations*. 2011: Cengage Learning.
26. Rivers, D.B. and G.A. Dahlem, *The Science of Forensic Entomology*. 2013: Wiley.
27. Puff, H., *Sodomy in Reformation Germany and Switzerland, 1400-1600*. 2003: University of Chicago Press.

28. von Bar, L., *A History of Continental Criminal Law*. 1999: Lawbook Exchange.
29. Madea, B., *Handbook of Forensic Medicine*. 2014: Wiley.
30. Lyle, D.P., *Howdunit Forensics*. 2008: F+W Media.
31. Mohan Kumar, T.S., et al., *Early adipocere formation: a case report and review of literature.* J Forensic Leg Med, 2009. **16**(8): p. 475-7.
32. Aufderheide, A.C., *The Scientific Study of Mummies*. 2003: Cambridge University Press.
33. Osinowo, T.O., et al., *BMAT and UKCAT Uncovered: A Guide to Medical School Entrance Exams*. 2011: Wiley.
34. Nickell, J. and J.F. Fischer, *Crime Science: Methods of Forensic Detection*. 1999: University Press of Kentucky.
35. Severinghaus, J.W., *Fire-air and dephlogistication. Revisionisms of oxygen's discovery.* Adv Exp Med Biol, 2003. **543**: p. 7-19.
36. Leicester, H.M. and H.S. Klickstein, *A Source Book in Chemistry, 1400-1900*. 1952: Harvard University Press.
37. Muljadi, E.P., *Arsenic*. Paul Muljadi.
38. Ramsland, K., *Forensic Science of CSI*. 2001: Penguin Group US.
39. Vidocq, E.F., *Memoirs of Vidocq: Principal Agent of the French Police Until 1827 [and Now Proprietor of the Paper Manufactory at St. Mandé]*. 1834: E.L. Carey & A. Hart.
40. Siegel, J.A. and K. Mirakovits, *Forensic Science: The Basics, Second Edition*. 2006: Taylor & Francis.
41. Conser, J.A., et al., *Law Enforcement in the United States*. 2011: Jones & Bartlett Learning, LLC.
42. Newton, M., *The Encyclopedia of Unsolved Crimes*. 2004: Facts On File, Incorporated.
43. Bertomeu-Sanchez, J.R., *Popularizing controversial science: a popular treatise on poisons by Mateu Orfila (1818).* Med Hist, 2009. **53**(3): p. 351-78.
44. Orfila, M.J.B., *Traité des poisons tirés des règnes minéral, végétal et animal, ou Toxicologie générale, considérée sous les rapports de la physiologie, de la pathologie et de la médicine légale*. 1818: Crochard.
45. Newcomb, S., *The World in a Crucible: Laboratory Practice and Geological Theory at the Beginning of Geology*. 2009: Geological Society of America.
46. Vander Voort, G.F., *Metallography, Principles and Practice*. 1984: ASM International.
47. Moore, L.J., *Sperm Counts: Overcome by Man's Most Precious Fluid*. 2007: NYU Press.
48. Tilstone, W.J., K.A. Savage, and L.A. Clark, *Forensic Science: An Encyclopedia of History, Methods, and Techniques*. 2006: ABC-CLIO.
49. Hayes, A.W., *Principles and Methods of Toxicology, Fifth Edition*. 2007: Taylor & Francis.
50. Jäger, J., *Photography: a means of surveillance? Judicial photography, 1850 to 1900.* Crime, Histoire & Sociétés, 2001. **5**(1): p. 27-51.
51. Spencer, F., *History of Physical Anthropology*. 1997: Garland Pub.
52. Rafter, N.H., *The Origins of Criminology: A Reader*. 2009: Taylor & Francis.
53. Roth, M., *Crime and Punishment: A History of the Criminal Justice System*. 2010: Cengage Learning.
54. Fifer, B. and M. Kidston, *Wanted!: Wanted Posters of the Old West and Stories Behind the Crimes*. 2003: Farcountry Press.
55. Girard, J.E., *Criminalistics: Forensic Science, Crime, and Terrorism*. 2013: Jones & Bartlett Learning.
56. Dutelle, A.W., *An Introduction to Crime Scene Investigation*. 2013: Jones & Bartlett Learning.
57. Houck, M.M., *Science Versus Crime*. 2009: Facts On File, Incorporated.
58. McElreath, D.H., et al., *Introduction to Law Enforcement*. 2013: Taylor & Francis.
59. Landsteiner, K., *The Specificity of Serological Reactions*. 1990: Dover Publications, Incorporated.

60. Mayr, W.R. and G.J. Thorbecke, *Epitope Recognition Since Landsteiner's Discovery: 100 Years Since the Discovery of Human Blood Groups ; with 11 Tables*. 2002: Springer Berlin Heidelberg.
61. Yount, L., *Forensic Science: From Fibers to Fingerprints*. 2007: Facts On File, Incorporated.
62. Krude, T., *DNA: Changing Science and Society*. 2004: Cambridge University Press.
63. Jeffreys-Jones, R., *The FBI: A History*. 2007: Yale University Press.
64. Theoharis, A.G., *The FBI: A Comprehensive Reference Guide*. 1999: Oryx Press.
65. Osborn, A.S., *Questioned Documents*. 1929: Boyd Printing Company.
66. Nickell, J., *Detecting Forgery: Forensic Investigation of Documents*. 2005: University Press of Kentucky.
67. Chisum, W.J. and B.E. Turvey, *Crime Reconstruction*. 2011: Elsevier Science.
68. Trimm, H.H., *Forensics the Easy Way*. 2005: Barron's.
69. Fridell, R., *Forensic Science*. 2007: Lerner Publications Company.
70. Kubic, T.A., *Dr. Walter C. McCrone--his contributions to environmental microscopy.* J Forensic Sci, 2004. **49**(2): p. 277-9.
71. Peterson, J.L., *Dr. Walter McCrone's contributions to microscopy workshops and the certification of criminalists.* J Forensic Sci, 2004. **49**(2): p. 267-9.
72. Feder, K.L., *Encyclopedia of Dubious Archaeology: From Atlantis to the Walam Olum: From Atlantis to the Walam Olum*. 2010: ABC-CLIO.
73. Butler, J.M., *Advanced Topics in Forensic DNA Typing: Methodology*. 2011: Elsevier/Academic Press.
74. Zalman, M. and J. Carrano, *Making Justice: Making Justice*. 2013: Taylor & Francis.
75. Olshaker, M. and J. Douglas, *Journey Into Darkness*. 2012: Random House.
76. Jebb, J.F., *True Crime: Virginia: The State's Most Notorious Criminal Cases*. 2011: Stackpole Books.
77. Butler, J.M., *Fundamentals of Forensic DNA Typing*. 2009: Elsevier Science.
78. Sobrino, B., M. Brion, and A. Carracedo, *SNPs in forensic genetics: a review on SNP typing methodologies.* Forensic Sci Int, 2005. **154**(2-3): p. 181-94.
79. Rudin, N. and K. Inman, *An Introduction to Forensic DNA Analysis, Second Edition*. 2001: Taylor & Francis.
80. Semikhodskii, A., *Dealing with DNA Evidence: A Legal Guide*. 2007: Taylor & Francis.
81. Justice, N.S.S., *DNA Analyst Training*, 2005.
82. McClintock, J.T., *Forensic Analysis of Biological Evidence: A Laboratory Guide for Serological and DNA Typing*. 2014: Taylor & Francis.
83. Moeller, K.E., K.C. Lee, and J.C. Kissack, *Urine Drug Screening: Practical Guide for Clinicians.* Mayo Clinic Proceedings. **83**(1): p. 66-76.
84. Fisher, B.A.J. and D.R. Fisher, *Techniques of Crime Scene Investigation, Eighth Edition*. 2012: Taylor & Francis.
85. Kobilinsky, L., *Forensic Chemistry Handbook*. 2011: Wiley.
86. Becker, R.F. and A.W. Dutelle, *Criminal Investigation*. 2013: Jones & Bartlett Learning.
87. Mozayani, A. and C. Noziglia, *The Forensic Laboratory Handbook Procedures and Practice*. 2010: Humana Press.
88. Gunn, A., *Essential Forensic Biology*. 2011: Wiley.
89. Thompson, R. and B.F. Thompson, *Illustrated Guide to Home Forensic Science Experiments: All Lab, No Lecture*. 2012: O'Reilly Media, Incorporated.
90. Vennemann, M., et al., *Sensitivity and specificity of presumptive tests for blood, saliva and semen.* Forensic Sci Med Pathol, 2014. **10**(1): p. 69-75.
91. Goodwin, W., A. Linacre, and S. Hadi, *An Introduction to Forensic Genetics*. 2011: Wiley.
92. Patel, G. and A. Hopwood, *An evaluation of luminol formulations and their effect on DNA profiling.* Int J Legal Med, 2013. **127**(4): p. 723-9.
93. Virkler, K. and I.K. Lednev, *Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene.* Forensic Science International. **188**(1): p. 1-17.
94. BeMiller, J.N. and R.L. Whistler, *Starch: Chemistry and Technology*. 2009: Elsevier Science.

95. Myers, J.R. and W.K. Adkins, *Comparison of Modern Techniques for Saliva Screening*.* Journal of Forensic Sciences, 2008. **53**(4): p. 862-867.
96. Seidl, S., R. Hausmann, and P. Betz, *Comparison of laser and mercury-arc lamp for the detection of body fluids on different substrates.* Int J Legal Med, 2008. **122**(3): p. 241-4.
97. Vandenberg, N. and R.A. van Oorschot, *The use of Polilight in the detection of seminal fluid, saliva, and bloodstains and comparison with conventional chemical-based screening tests.* J Forensic Sci, 2006. **51**(2): p. 361-70.
98. Willott, G.M., *An Improved Test for the Detection of Salivary Amylase in Stains.* Journal of the Forensic Science Society, 1974. **14**(4): p. 341-344.
99. Whitehead, P.H. and A.E. Kipps, *A test paper for detecting saliva stains.* J Forensic Sci Soc, 1975. **15**(1): p. 39-42.
100. Old, J.B., et al., *Developmental Validation of RSID™-Saliva: A Lateral Flow Immunochromatographic Strip Test for the Forensic Detection of Saliva.* Journal of Forensic Sciences, 2009. **54**(4): p. 866-873.
101. Stark, M.M., *Clinical Forensic Medicine: A Physician's Guide*. 2011: Humana Press.
102. Moeller, K.E., K.C. Lee, and J.C. Kissack, *Urine Drug Screening: Practical Guide for Clinicians.* Mayo Clinic Proceedings, 2008. **83**(1): p. 66-76.
103. Satyanarayana, U., *Biochemistry*. 2014: Elsevier Health Sciences APAC.
104. Elkins, K.M., *Forensic DNA Biology: A Laboratory Manual*. 2012: Academic Press.
105. Ponten, F., et al., *A global view of protein expression in human cells, tissues, and organs.* Mol Syst Biol, 2009. **5**: p. 337.
106. Latchman, D., *Gene Regulation*. 2007: Taylor & Francis.
107. Richard, M.L., et al., *Evaluation of mRNA marker specificity for the identification of five human body fluids by capillary electrophoresis.* Forensic Sci Int Genet, 2012. **6**(4): p. 452-60.
108. Bowden, A., R. Fleming, and S. Harbison, *A method for DNA and RNA co-extraction for use on forensic samples using the Promega DNA IQ™ system.* Forensic Science International: Genetics, 2011. **5**(1): p. 64-68.
109. Juusola, J. and J. Ballantyne, *Multiplex mRNA profiling for the identification of body fluids.* Forensic Science International. **152**(1): p. 1-12.
110. Roeder, A. and C. Haas, *mRNA profiling using a minimum of five mRNA markers per body fluid and a novel scoring method for body fluid identification.* International Journal of Legal Medicine, 2013. **127**(4): p. 707-721.
111. Park, J.L., et al., *Identification of body fluid-specific DNA methylation markers for use in forensic science.* Forensic Sci Int Genet, 2014. **13C**: p. 147-153.